Stem Cells in Toxicology and Medicine

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Dedication

I lovingly dedicate this book to:
My parents, Gopinath and Ichhamoni, for their gifts of life, love and living example.
My wife, Jharana, for her life-long friendship, love and support as well as for her patience and understanding about the long hours spent at home on planning, writing and editing this book.
My children, Megha, Sudhir, and Subir, for their love and care.

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Preface

Stem cells are undifferentiated cells in multicellular organisms capable of growing into various differentiated cell types. In recent years they have become an important research tool in biology, medicine and toxicology leading to a rapidly developing new scientific discipline. This monograph focuses on their use in toxicology and medicine at a level designed to take readers to the frontiers of research in this specialized area. The importance of this field of research is evidenced by the increasing number of articles published each year. This rapid development requires new means to report the results of ongoing studies. The contributions presented in this monograph represent a collaborative effort by international experts working in this emerging field of science. The main aim of this book is to present state-of-the-art information on stem cells in one place. Therefore, I sincerely hope that this book will provide a comprehensive and authoritative source of current information on stem cells and prove useful to the investigators and students working in this scientific discipline throughout the world for years to come. It is my hope that the information presented in this book will serve as a stimulus to them. Also it is my hope that it will be of interest to a variety of other scientific disciplines including pharmacology, food, drug, and environmental sciences. In addition, this book should be of interest to the safety assessors and regulators of food, drug, environment, agriculture, and consumer products.

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Part I

1
Introduction

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Stem cells are the mothers of all cells in multicellular organisms. They have the potential to become any other type of cell in the body. They are undifferentiated cells of the same family capable of dividing throughout life, generating new highly differentiated cells of unlimited potency. Because of their unique regenerative abilities, they can serve as an internal repair system to replenish damaged or dead cells in many tissues. Therefore, they have attracted increasing amounts scientific attention for their potential use in biomedical applications.

The studies by McCulloch and Till published in 1963 (Becker et al., 1963; Siminovitch et al., 1963) gave birth to modern stem cell research. Over a period of approximately half a century, there was exponential growth in this developing new area of scientific research. Stem cells have the capacity to grow in culture continuously in an undifferentiated state renewing themselves to more specialized differentiated cells. Therefore, they have become a very important and useful *in vitro* research tool in toxicology and medicine. They can be used as excellent *in vitro* models for predictive toxicity screening of chemicals and new drugs. Thus, the study of stem cells is a new developing scientific discipline and their use in toxicology and medicine is unlimited.

It is becoming increasingly clear from the rate of publications that developments in the use of stem cells in toxicology and medicine are moving so rapidly that new means are needed to report the current status of this new active area of research. As the Editor of this monograph *Stem Cells in Toxicology and Medicine*, it gives me great pride and pleasure to introduce this unique book that encompasses many aspects of stem cell research never published together before. It is only recently that this exciting area of research has attracted the attention of toxicologists. This book deals with information on stem cells at a level designated to take the reader to the frontier of research in this specific new developing scientific discipline. It is expected that stem cell research, actively pursued throughout the world, will lead to major discoveries of fundamental importance and of great clinical significance. This monograph brings together the ideas and work of investigators of international reputation who have pioneered in this exciting area of research in toxicology and medicine.

Stem Cells in Toxicology and Medicine, First Edition. Edited by Saura C. Sahu.

The book provides up-to-date information as well as new challenges in this exciting area of research. This book reflects the remarkable developments in the stem cell technology in recent years. New ideas and new approaches are being brought to bear on explorations of the role played by these unique cells in toxicology and medicine. Therefore, exciting times lie ahead for the future of stem cell research. I sincerely hope that the book will provide authoritative information as well as new ideas and challenges in this area of research for stimulating the creativity of investigators actively engaged in this rapidly developing new scientific discipline.

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2

Application of Stem Cells and iPS Cells in Toxicology

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2.1 Introduction

Fertilization of an oocyte by a spermatozoid, or in general terms, fertilization of the female gamete by the male gamete results in the genesis of the basic unit in the development of an organism, the embryo. The primordial unicellular embryo will, after several rounds of division, develop into a multicellular organism and ultimately into an offspring resembling its progenitors in structure and function. At the molecular level, embryonic development is governed by the expression and interaction of a specific set of genes, certain proteins and peptides, and the signaling of growth factors. In other words, embryonic development is a complex process, which involves a vast but specific genetic network corresponding to its different stages.

Early observations made in the sea urchin (Driesch, 1892, 1893, 1894; Gilbert, 2000; Roux, 1888; Roux, 1894) demonstrated that splitting a two-cell embryo into single cells resulted in the emergence of two fully developed organisms. Hans Spemann repeated these experiments in the salamander two-cell embryo, ultimately obtaining two fully developed "twins" (Gilbert, 2000; Spemann, 1921). These early experiments demonstrated the totipotency of the early embryonic cells and the retention of certain "information," which allows for the development of an organism. This information is progressively lost as the embryo develops and its cells differentiate and reach more specific roles (Gilbert, 2000; Spemann, 1921). However, the question remains, what is the stage at which cells lose this potential?

Further experimentation revealed that cells derived from teratomas, or from mouse blastocysts inner cell mass (ICM) cultured upon a suitable fibroblast feeder layer, continue to proliferate without overt differentiation

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and remain totipotent (Evans and Kaufman 1981; Martin 1981). Consequently, when these Embryonic Stem Cells (ESc) are removed from the differentiation-inhibitory influence of the feeder cells, or equivalent, they will spontaneously differentiate into developing embryo-like structures of increasing complexity, or embryoid bodies (EBs) (Doetschman et al., 1985; Martin and Lock, 1983). Embryoid bodies thus, enable researchers to study different aspects of early embryonic development. Indeed, with the advent of novel molecular biology techniques, it has been demonstrated that these cells are competent for gene targeting via homologous recombination or site directed mutagenesis. They are competent for the generation of genetically modified model systems, including mouse models for the study of developmental embryonic formation, diseases driven by genetic mutation, transcriptional regulation, or molecular toxicological effects (Boch, 2011; Bradley et al., 1984; Capecchi, 1980; Hendel et al., 2015; Koller et al., 1989; Mali et al., 2013; Smithies et al., 1985; Thomas and Capecchi, 1990).

2.2 Significance

Understanding stem cells and iPS cells in terms of their dynamic differential molecular signatures opens new avenues for the study of the effects of pharmaceutical chemicals at the cellular, physiological, and ultimately the molecular levels. Furthermore, stem cells and pluripotent stem cells present clear advantages compared to other model systems currently used for testing pharmaceutical compounds. The traditional methods for compound testing include the use of primary cells and/or live models, such as mouse or zebrafish (Parasuraman, 2011; Sipes et al., 2011). Primary cells from any organ present the difficulty of the initial isolation, quantity and quality per isolated batch, and different proliferation capacities, making industrial scalability a difficult process. Furthermore, genetic background variability leads to inconsistent cellular responses which in turn lead to a broad array of results, interpretations and conclusions (McGivern and Ebert, 2014). Stem cells, and especially induced pluripotent stem cells, circumvent the majority of these obstacles. Indeed, isolation of somatic tissue cells, whether it is skin cells, blood cells, or epithelial cells harvested from urine (Zhao et al., 2013), is relatively easy, non-invasive, and most importantly, patient specific. Harvested cells are genetically homogenous, whether these are from non-diseased or diseased patients, allowing for direct experimental testing; cellular responses are consistent and variability is minimal. Pluripotent stem cells possess a high self-renewal capacity and proliferate unlimitedly. Indeed, recent reports indicate that high cellular passage (higher than 60) have no effect in proliferation rates or in differentiation potential (Burridge et al., 2014). Pluripotent stem cells that possess unlimited proliferation, that is, cells that surpass the Hayflick limit (Hayflick, 1965; Hayflick and Moorhead, 1961), have been reported to be highly related to telomerase activity and hTERT expression necessary for telomere length maintenance (Huang et al., 2011). This unlimited proliferation indicates that scalability necessary for industrial testing is achievable (Couture, 2010). Furthermore, iPS cell growth and differentiation *in vitro* using cell culture techniques and materials amount to a fraction of the cost invested in live animal models (Burridge et al., 2014). Finally, the most important characteristic of iPS cells is related to their specific human physiological identity. Historically, pharmaceutical testing has been performed in live animal models, such as mice, zebrafish, rats and pigs; however, iPS cells are of human origin, and the molecular, physiological and cellular responses are the most adequate for molecular testing, especially in studies related to electrical conduction such as neuronal or cardiac. It is known, for example, that cardiac ion channels which rectify the cardiac rhythm are different in rats from those in humans, thus, extrapolation of results is inconclusive (Grant, 2009; Han et al., 2010). Finally, iPS cells are derived from differentiated adult human tissue and not from a surplus of fertilized human embryos; therefore, iPS cells are not in conflict with any religious or humanistic ethical principles (McGivern and Ebert, 2014).

As noted previously, the understanding of stem cells, in terms of cell renewal and differentiation capacity, and the acquired knowledge of their dynamic differential molecular signatures, leads to new routes of research

to investigate the effects of pharmaceutical chemicals at the cellular, physiological, and molecular levels. Furthermore, iPS cells are physiologically relevant, genetically homogenous, amount to a considerably lower cost and are free of ethical conflicts. Taken all together, these characteristics make stem cells and pluripotent stem cells a highly beneficial research and industrial platform, especially for studies aimed at pharmacodynamic/kinetic outcomes and toxicological screenings (McGivern and Ebert, 2014).

2.3 Stem Cell (SC) Classification

Since their discovery, stem cells have been the object of intense study. As noted above, stem cells were initially identified and characterized as immortal cells isolated from teratomas (cancerous embryoid-like bodies) (Martin, 1981). However, further research demonstrated that stem cells are varied and tissue-context dependent. Indeed, embryonic stem cells are found and isolated from the inner cell mass (ICM) of the early mouse blastocyst and from the fetal umbilical cord blood (Evans and Kaufman, 1981). Additionally, stem cells can also be found in skeletal muscle, as a satellite cell population (Hawke and Garry, 2001); the bone marrow, as hematopoietic progenitor cells (Sieburg et al., 2006); the small intestine, as crypt cells (Barker, 2014). In other words, stem cells are found in every tissue/organ and display a differential potential for self-renewal and regenerative capacity.

Thus, by their origin, stem cells are classified as: (1) Embryonic stem cells, (2) Fetal stem cells, and (3) Adult stem cells. By their potential of self-renewal and regenerative capacity, stem cells are classified as: (1) Totipotent, (2) Pluripotent, (3) Multipotent, (4) Oligopotent, and (5) Unipotent (Bissels et al., 2013).

In this classification we find the concept of Totipotent stem cells (toti-, totus = all; -potent = power). These are capable of developing a whole embryo, capable of differentiating into any type of cell or tissue of the mature organism, and are able to generate embryonic tissues and extraembryonic membranes. However, as we advance our knowledge, there appears to be some controversy regarding terminology. Recently, the term of totipotent is presented as the capacity of one cell to produce a fertile adult organism. However, and interestingly, a new concept of "plenipotent" appears, in order to specify the capacity of stem cells to produce an adult organism, albeit those cells lack the capacity to organize themselves in a coherent body plan, thus, totipotency and plenipotent would be reserved only for organism and for cells respectively (Condic, 2013). The recent confusion about totipotency has led some to propose a new definition, with one author suggesting the term "totipotent" again be reserved only for organisms, while stem cells that produce all cell types but do not organize them into a coherent body plan would be referred to as "omnipotent" (Denker, 2004).

Pluripotent (plurimus- = many, -potent = power) stem cells are descendants of totipotent stem cells. These cells are capable of differentiating into all embryonic and adult cell types except extraembryonic membranes. Pluripotent stem cells are available as undifferentiated embryonic stem cells (ESCs) and can be cultured *in vitro* as permanent lines. Specifically, human embryonic stem cells (hESCs), established from surplus embryos after *in vitro* fertilization, are currently available with more than a thousand individual cell lines (Löser et al., 2010). Recently, a new class of stem cells, human induced pluripotent stem cells (hiPSc), have been generated from adult reprogrammed somatic cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). These iPS cells are capable of generating a viable, fertile fully-grown organism; thus, iPS cells could be classified as adult pluripotent stem cells.

Similarly, multipotency, oligopotency, and unipotency refer to the progressively reduced capacity of a stem cell to differentiate into several, few, or only one type of cell. A clear example of this graded potency is observed in the bone marrow (Hematopoietic) progenitor cell tree which generates primarily myeloid and lymphoid cell progenitors, and ultimately will produce red and white blood cells. These hematopoietic stem cells are considered multipotent; however, daughter cells progressively lose the capacity of self-renewal and differentiation diversity as these cells differentiate and assume more specific roles.

Thus, a myeloblast, or a small lymphocyte, could be considered oligopotent and a monocyte and B-lymphocyte could be considered unipotent (Bissels et al., 2013; Sieburg et al., 2006).

2.4 Stem Cells and Pharmacotoxicological Screenings

The use of *in vitro* cell cultures to predict *in vivo* effects is troubled with difficulties related to the evaluation of pharmacokinetics and pharmacodynamics; that is, the detection of the absorption and systemic metabolism observed in a relevant organ *in vivo*, being challenging to predict in an *in vitro* model. Although a deeper knowledge is required concerning the characteristics and function of stem cells, and iPS cells, these system platforms are considered a potentially highly beneficial research tool to be used in various disciplines, including toxicology.

It has been reported that primary cells are often more sensitive to certain drugs or chemicals than established commercial cell lines. Therefore, the use of undifferentiated cell lines can provide more relevant and predictive toxicity results. Moreover, recent studies have demonstrated that induced pluripotent stem cells (iPSCs) can recapitulate the phenotype of several known diseases, making iPS cells a promising cell source for predictive drug screening (Wang et al., 2014; Zeevi-Levin et al., 2012).

The toxicological evaluation of chemicals using embryonic stem cells has only recently been considered as a screening and pharmacological platform. As an example, stem cells are being utilized to evaluate the risk of a chemical as Perfluorooctane sulfonate (PFOS). PFOS is commonly used as a simple salt (such as potassium, sodium or ammonium), or is incorporated into larger polymers, as a stain repellent and fabric protector. Xu and colleagues evaluated the effect of PFOS, using mEBs (mice embryoid bodies) cultured by the suspension method. By using mice embryoid bodies, as a risk assessment tool, the authors concluded that PFOS poses potential risks to early development by downregulating the expression of pluripotency and stem cell molecular markers induced cell apoptosis by changing lysosomal membrane permeabilization and therefore blocking autophagy (Xu et al., 2013, 2015; Yao et al., 2014).

2.5 Industrial Utilization Showcases Stem Cell Technology as a Research Tool

Similarly, other chemicals have been tested utilizing the mEBs platform. Indeed, arsenic, as well as Estrogen E2, has been shown to disturb and interfere with mEB differentiation by downregulating the expression of genes involved in the differentiation of the three embryonic cell layers, such as Hepatocyte Nuclear Factor, brachyury, and cardiac actin. These results show that mEBs platforms are now considered an excellent system for the study of pharmacological developmental risks (Flora and Mehta, 2009; Hye-Ryeon et al., 2015; Kim et al., 2013).

Despite having been proven to be an excellent platform, mEBs are still of limited use given that observed defects are only related to early developmental stages, such as aberrant organ formation or congenital defects; the former will result in embryonic death, the latter would require surgical interventions. However, pharmacologically manageable and treatable diseases, such as chronic pain or cancer, will result in unwanted and unpredicted secondary effects such as high pressure-induced cardiac arrest or heart failure (Renet et al., 2015).

Secondary cardiotoxicity has recently become an important issue in drug development, as many newly synthesized molecules are causing unexpected cardiac damage, and are altering physiological or biochemical parameters. Indeed, some molecules act upon cardiomyocyte receptors, therefore changing the cell membrane potential leading to arrhythmias and sudden death (Hou et al., 2015). Thus, the development of an investigative tool, which mimics the physiological conditions of the human cell, and which allows for

testing of cell behavior upon drug dosage, is a major necessity. In this regard, stem cells/iPS cells-derived differentiated cells are showing a very promising future.

As noted, the use of hPSCs as an independent cell system and as an early predictive tool for the detection of pharmacological effects in humans has emerged as a powerful alternative to live animal systems. The properties of hPSC-derived cardiomyocytes are similar to their *in vivo* cardiac counterparts. For example, hPSCs express the major cardiac ion channels, produce the three major cardiac action potentials, and respond in identifiable and anticipated ways to known chemicals (Caspi et al., 2008; Liang et al., 2010). Thus, the use of PSCs is becoming advantageous in safely evaluating cardiac pharmacology. Consequently, the development of successful new tools, which mimic complex human physiological conditions, will potentially bridge the gap between drug development and early clinical trial stages.

Moreover, physiological response is an important aspect to consider when assessing drug efficiency. Human patients respond differently to a given drug due to various factors, including genetic predisposition, age, ethnicity, and so on. New strategies to palliate those factors will contribute to a more effective drug discovery and to a more refined personalized medicine, thus improving drug efficiency. Indeed, by analyzing hPSC in terms of drug response, both multi electrode arrays (MEA) and patch clamping results revealed that hPSC-derived cardiomyocytes show an anticipated and consistent dose-responsive effect on beat rate and electrophysiological changes across a set of 43 compounds (Dick et al., 2010) and across 19 different compounds (Laposa, 2011).

A fast and efficient drug screening platform contributes to the modernization of personalized medicine. Currently, many public and private laboratories are using hPSC-derived tissues for molecular screening, focusing on the identification of new targets and drug molecules (Kim and Jin, 2012; Yahata et al., 2011). The improved biological properties of hPSC-derived tissues become even more useful when combined with an increasing number of sophisticated tools to introduce precise genetic alterations, such as CRISPR, TALENs, BAC-induced recombination, and so on (Bedell et al., 2012; Doyon et al., 2008; Gaj et al., 2013; Hwang et al., 2013). Furthermore, human PSC-derived tissues might be engineered to replicate diseases, to express factors that enable differentiation or maturation, or to introduce biomarker-reporting constructs (Chen et al., 2014; Wang et al., 2014; Yahata et al., 2011).

Taken together, human PS cells are showing more prominence in research fields as important as cardiology and toxicology and are being considered as a potential screening tool for the identification of toxicological effects, for pharmacology safety, and for the study of differentiation defects.

2.6 Multipotent Stem Cells (Adult Stem Cells) Characteristics and Current Uses

The classification system of stem cells mentioned above leads us to describe the class of multipotent stem cells, or adult stem cells. These cells are present in tissues with a relatively high regenerative capacity, and possess several common characteristics including differentiation capacity, self-renewal, and clonogenicity.

Multipotency is described as the capacity or potential to differentiate into multiple but limited cell types. As noted previously, the multipotent blood stem cells (hematopoietic line), are able to replenish the niche of stem cells and also differentiate into the several blood cell lineages, such as myeloid and lymphoid lineages. However, this population cannot differentiate into other cells types such as neurons, bone or other non-blood cell types. Stem cells will generate two daughter cells, which could be two stem cells or two differentiating cells (symmetric division), or a stem cell and a differentiating cell (asymmetric division) (Harandi and Ambros, 2015; Morrison and Kimble, 2006). The proper regulation of stem cell asymmetric versus symmetric cell division is fundamental for the correct formation of developmental patterns, the determination of cellular fates, and for normal tissue growth and homeostasis.

Clonogenicity is understood as the capacity of one cell to divide and create a group of cells or colony in cell culture. Indeed, Buzhor and colleagues showed that NCAM1+ cells exhibit robust clonogenicity, mesenchymal differentiation, sphere-formation capacity, and retain the ability to produce renal epithelial tissue using a limiting dilution assay (Buzhor et al., 2013).

Regulation of stem cell differentiation capacity, clonogenicity, and self-renewal capacity would yield an extended knowledge of the effect that chemicals can wield upon them. Indeed, differentiation patterns are affected by the exposure to chemicals widely used in industrial and chemical manufacturing, such as benzene. Epidemiological studies and case reports have suggested a close relationship between occupational exposure to benzene and hematotoxicity and various types of leukemia (IARC, 1982). Similarly, Zhu and colleagues studied the effect of hydroquinone (HQ) (the main derivative of benzene metabolism) in mice. Hydroquinone exposure results in a higher mutation rate and modification of the murine Cyp4f18, a gene orthologous to the human CYP4F3 gene (a cytochrome P450 gene involved in metabolism and synthesis of cholesterol), leading to malignant transformation of blood cells. Also, concentration-dependent HQ-induced up-regulation of Cyp4f18 in embryonic yolk sac hematopoietic stem cells (YS-HSCs) and adult bone marrow hematopoietic stem cells (BM-HSCs) results in decreased proliferation, lower colony formation potential, and reduced differentiation rates, while increasing the apoptotic rate. These results are evidence supporting the notion that benzene and its metabolites target hematopoietic stem cells (HSCs), thus causing differentiation pattern defects, blood toxicity, and cancer (Zhu et al., 2013).

Consequently, it is understood that the system's internal equilibrium is of paramount importance as it has been vastly reported. In normal tissue homeostasis, stem cell division is thought to be infrequent and to involve an asymmetric daughter-cell-fate decision, in which one daughter remains as a SC while the second partially differentiates into a progenitor cell with short-term growth potential (Harandi and Ambros, 2015; Morrison and Kimble, 2006). Drugs, chemical or environmental pollutants could interfere with this equilibrium, and therefore might modify tissue homeostasis. Interestingly, the effects that environmental pollutants have upon the system can target not only resident cells themselves, but can also interact with the cellular microenvironment. Specific parameters such as time of exposure, concentration levels, and chemical lifetime stability, will lead to changes in extracellular ion concentration, oxygen levels, water surface tension, extracellular signal molecules, metal chelation, and so on. In addition to these factors, other intrinsic factors, such as age, genetic predisposition, cancer history, and lifestyle could create a context that may promote the changes in cell homeostasis and consequently lead to tissue damage (Casey et al., 2015).

2.7 Mesenchymal Stem Cells (Adult Stem Cells)

In the last few years, mesenchymal stromal cells (MSCs) have become a fast growing field of interest in industry and biomedical science. Mesenchymal stem cells are classified as adult stem cells, capable of self-renewal and differentiation into multiple mesodermal cell lineages including cartilage, adipose tissue, and bone. These cells are characterized by their ability to adhere to plastics under standard cell culture conditions, and can be identified by surface markers such as, CD44, CD73, CD90, CD105; however, MSCs do not express CD45, CD34, and CD14 (Natunen et al., 2013). Mesenchymal stem cells are present in relatively low numbers in various tissues such as bone marrow, adipose, muscle, and at minimal levels in many other tissues. Hematopoietic stem cells are considered a subgroup of MSCs, which supports the hematopoietic differentiation process (Dazzi et al., 2006). Isolated MSCs from these tissues, such as adipose tissue-derived multipotent stromal cells (AT-MSC), are utilized in immune modulatory research as an alternative to the gold standard bone marrow-derived MSCs (BM-MSCs). This methodology is reported to be a safer approach, due to minimal risk of isolation, and furthermore, larger amounts of AT-MSC can be obtained compared to

BM-MSC. Mesenchymal stem cells, and in particular BM-MSC and AT-MSC, share a similar immune phenotype and it has been reported that both present similar high multi-lineage differentiation capacity *in vitro* (Melief et al., 2013).

Despite their usefulness, mesenchymal cells are not extensively utilized. However, recent reports describe their favorable applications in the toxicology research field. Indeed, toxicological effects of pet food ingredients were assessed in canine bone marrow-derived mesenchymal stem cells (BMSC) and in enterocyte-like cells (ELC). Exposure of BMSC and ELC to these ingredients for 24 h induced metabolic dysfunction observed as dysregulation of CYP450, mitochondrial energy metabolism, changes in β-oxidation, and so on, leading to changes in cell viability. The improvement in the methodology characterized in this new *in vitro* BMSC and ELC system reported by Ortega and colleagues (Ortega et al., 2015), shows a high potential value for MSCs as a tool to investigate the overall toxic or irritant effect of food ingredients on rapidly dividing or early differentiated cells.

Furthermore, not only were the BMSC and ELC systems, reported by Ortega, used to detect cell viability, but also BM-MSC and ASCs were employed to test collateral effects of anti-lymphocyte serum (ALS) or tacrolimus (an immunosuppressive drug) on cell viability and behavior modification *in vitro* (Tsuji et al., 2015). Thus, the mesenchymal stem cell platform usefulness is multi-faceted; however, our understanding and knowledge are still incomplete and far away from unlocking the biggest potential and benefits that these platforms could provide.

2.8 Hematopoietic Stem Cells (Adult Stem Cells)

Hematopoietic cells (MHCs), are considered to be multipotent stem cells, having the ability to differentiate into special circulating cells such as erythroid cells, granulocytes, macrophages, megakaryocytic, and lymphoid blood cells (Valeri et al., 2010). The use of this population of cells in research began approximately half a century ago, with bone marrow (BM) initially deployed to achieve donor-specific transplantation tolerance. This cell population was used in several animal models and was addressed extensively by a high number of authors. Importantly, early studies on embryonic mice and chickens demonstrated that acquisition of immunological tolerance is induced by cell/tissue graft transplants at embryonic stages (Billingham et al., 1953). These studies showed a cellular chimerism-induced tolerance response and resulted in a donor-specific transplantation tolerance. Furthermore, using adult animal models, total reconstitution of the hematopoietic system was achieved by combining BM transplant (BMT) following the preconditioning of the host by different regimens, including total body irradiation (Ildstad and Sachs, 1984), total lymphoid irradiation (Slavin et al., 1977), and anti-lymphocyte globulin (Caridis et al., 1973; Monaco et al., 1976).

Recent studies using hematopoietic cell lines show that HSCs present a high differentiation capacity and indicate that culture-expanded BMSCs are capable of differentiating into neural precursors, chondrogenic (Skreti et al., 2014), osteogenic, and adipogenic (Sayed et al., 2014) lineages, cardiomyocytes (Fukata et al., 2013; Hou et al., 2013), as well as hepatocytes (Brückner et al., 2013).

The high capacity of differentiation shown by this cellular line makes it a desirable new alternative in bioengineering and tissue regeneration. Indeed, the use of HSCs in oncology has shown that daunomycin, a known potent antitumor agent, induces dysregulation in the expression of certain histones, in addition to the modification of histone methylation patterns in a time-dose dependent manner, thus affecting chromatin condensation (Aramvash et al., 2012). Additionally, HSCs have recently been described as an alternative in toxicology as well.

Alternatively, a new and innovating technology has emerged known as "bone marrow-on-a-chip." With applications in pharmacology and toxicology, this methodology provides scientists with a new tool to test the effects of drugs, chemicals, or toxic agents on the whole bone marrow, albeit in a much

smaller scale (Torisawa et al., 2014). The model is described to support immunogenicity and immunotoxicity testing and long-term cultivation with repeated antigen stimulation. Recently, another variant of the mentioned methodology described as "organ-on-chip device" reconstitutes and sustains an intact, functional, living bone marrow. However, the implementation of these technologies in a large scale is still under revision, and the potential that these devices could offer to replace tests of acute systemic toxicity in animals is still under discussion. Nevertheless, these technologies are bringing us closer to the notion of *in vitro* recapitulation of human physiology, such as the simulation of the human immune system (Kim et al., 2015).

In order to implement these current methodologies fully, important parameters must be considered, such as tissue environment and tissue homeostasis. The response of a specific cell line in a tissue upon drug treatment, and even the influence after treatment by the cell lines adjacent to it, is described to follow a hierarchical organization, including cell-cell communication, signaling responses to external stimuli, and so on (Khetani and Bhatia, 2008; Tay et al., 2011). Bone-marrow-on-a-chip, a recent addition to the "organ-on-a-chip" field, is expected to bring forth a new breakthrough in research by offering a more controlled environment to study the tissue's physiological response; however, this is still a work in progress (Torisawa et al., 2014). Although the system appears as a revolutionary alternative in toxicology, currently, poor predictability of in vitro drug toxicity in terms of pharmacokinetic and pharmacodynamics (PK/PD) is a major weaknesses and a challenge to overcome. Pharmacokinetic refers to a time-dependent concentration of a substance in plasma, whereas pharmacodynamics refers to pharmacological effect of a drug in the system (Derendorf and Meibohm, 1999). By combining PK/PD results with mathematical modeling multiple studies are geared towards multiple-organ-on a-chip (MOC) devices to mimic *in vivo* physiological complexities and test, for example, for drug side effects. Indeed, Sung and colleagues have shown the efficacy of a model-on-a-chip by testing the lethality of the metabolism of Tegafur, an oral cancer prodrug, to 5-Fluorouracil, the lethal metabolite, on isolated tumor cells seeded on a hydrogel platform (Sung and Shuler, 2009). Thus, tissue/organ simulation combined with PK/PD studies may bring alternative approaches to procedures of drug development and biosafety.

2.9 Cardiotoxicity

During the process of drug development, one of the major concerns is the secondary cardiac toxicity and it is imperative that clinical treatments are tested exhaustively for early adverse effects. Currently, safety pharmacology studies, for the evaluation of new drug entities for potential cardiac liability, remain a critical component of drug development process. Predicting the side effects of drugs, remains one of the industry's greatest challenges, with a large percentage of new drugs failing in clinical studies due their cardiotoxic effect. The overall successful result rate from Clinical Phase 1 studies is only 11%, and 30% of these fail for safety reasons (Kola and Landis, 2004). Cardiotoxicity includes any one of several adverse events, such as arrhythmia, myocardial ischemia, myocardial necrosis, or hypotension. Moreover, drug-induced cardiac safety concerns might develop with different timing, acutely (during or shortly after treatment), chronically (weeks to months after treatment), or years later as a consequence of treatment (Doherty et al., 2015). There is rising interest in assessing cardiotoxicity in the early stages of drug discovery, eliminating potentially toxic compounds before further time and money is spent. The development of highly predictive *in vitro* assays suitable for high-throughput screening (HTS) is critical to fulfill this request. In the 1990s, eight non-cardiovascular drugs were withdrawn from clinical use because they prolonged the QT interval in the heart's electrical cycle resulting in ventricular arrhythmias and potentially unexpected death (Fermini and Fossa, 2003). Drug-induced cardiotoxicity may emerge not only as a functional change in electrophysiology, but also as a change in the structural integrity of cardiac tissue. In fact, direct structural damage and

cardiomyocyte death can contribute to develop several heart diseases as cardiomyopathy, myocardial infarction, and heart failure. Structural damages can often result in effects that take longer to manifest (e.g., usage of anthracyclines in pediatric patients) (Lipshultz et al., 1991) and/or are enhanced with combined treatment (e.g., Herceptin plus anthracyclines) (Seidman et al., 2002) making it difficult to assess them with currently available models. Due to cardiac liability issues, significant numbers of drugs under development are dismissed in the late preclinical and early clinical stages, increasing significantly the overall cost of the candidate drug brought to market (Valentin, 2010). Moreover, the limited availability of relevant cardiomyocyte models has hindered the development of rational toxicity tests. Force and Kolaja recently reviewed the limitations of the commonly used model species in the field (e.g., rodent vs. human). Morphology, metabolism, or electrophysiology cascades can confuse the translation of *in vitro* cardiotoxicity findings to humans (Force and Kolaja, 2011). However, the recent advances in stem cell technology and particularly in differentiating embryonic or induced-pluripotent stem cells have created a unique opportunity for providing physiologically/disease relevant models for preclinical safety assessment of compounds (Kamp and Lyons, 2009).

Thus, SC/iPSCs exposed to a defined culture and pre-conditioned culture media is currently utilized to obtain a desired cell's lineage by selective differentiation (such as cardiomyocytes). Further, SCs/iPS cells might be enriched and selected as pure populations. Additionally, these cells might be obtained from genetically engineered sources. All of these properties make SC/iPSCs a powerful approach for pharmacology and toxicity studies (Abassi et al., 2012; Denning and Anderson, 2008; Freund and Mummery, 2009; Kamp and Lyons, 2009; Kettenhofen and Bohlen, 2008). Indeed, recent research supports the idea of the valuable use of SC or iPSCs as a tool for both; structural (Pointon et al., 2013) and electrophysiological (Abassi et al., 2012; Guo et al., 2013) drug-induced toxicity assessment.

There are two main approaches to produce human pluripotent stem cell-derived cardiomyocytes (hPS-CM) detailed next:

1. From human embryonic stem cells (hES-CM).
2. From human induced pluripotent (hiPS-CM) stem cells.

The latter is most laborious but offers the advantages of being patient-specific or patient-cohort-specific. hPS-CMs derived from either of these approaches (Streckfuss-Bömeke et al., 2013) have the potential to replace animal ventricular CM's used to screen new compounds' cardiotoxic effects during the preclinical drug development process. Currently, the screening for drug cardiotoxic effects uses neonatal and adult ventricular CMs from many different animals like rat, mouse, and guinea pig. Although the outcomes of these screens do not fully predict human responses as the species responses differ, the use of hES-CMs or hiPS-CMs for cardiac toxicity screening presents some advantages:

1. Studies suggest that there are significant differences between species in drug-induced toxicity (Baillie and Rettie, 2011). Different species express different types of cardiac ion channel proteins, and relative levels of each channel type change from species to species. Therefore, the use of a human cell model will provide a clearer statement early in drug development on defining whether the drug will be cardiotoxic in humans or not, helping in compound selection and substantially reducing drug decline.
2. Higher cost efficiency. The usage of laboratory-produced hPS-CMs for cardiac toxicity screenings may reduce these costs by up to 50%.

In order to recruit the potential of stem cell-derived cardiomyocytes for *in vitro* preclinical safety screening and assessment, Abassi and colleagues in 2012 developed a microelectronic sensor-based system with the potential to determine the dynamic and rhythmic beating process of cardiomyocytes (Abassi et al., 2012).

On the other hand, fast kinetic fluorescence imaging systems, can estimate the effect of pharmacological compounds on the beating rate of stem cell derived cardiomyocytes. Thus, iPSC-derived human cardiomyocytes are demonstrated to be useful as a model system to measure the impact of pharmacological compounds on the beat rate of spontaneously contracting cardiomyocytes. Therefore, developing and promoting assays based on human cardiomyocytes derived from stem cell sources greatly accelerate the progress of new chemical entities and improve drug safety by offering more biologically relevant cell-based models than currently available.

Stem cell-derived cardiomyocytes expression of specific ion channel proteins leads to a demonstrated spontaneous mechanical and electrical activity analogous to native cardiac cells, which ultimately results in a physiological change in intracellular Ca^{2+}, a common and useful read-out for cardiomyocyte contractility. iPSc-CMs cellular assays employing calcium sensitive dyes to monitor variations in Ca^{2+} concentration help to determine specific parameters, such as beat rate and amplitude, which are useful for the estimation of drug candidate efficacy and safety prior to clinical studies in humans. Recently, cardiomyocyte contraction rate documentation by using automated microscopy to monitor changes in concentration of Ca^{2+} has been utilized to assess the effect of positive and negative chronotropic agents and cardiotoxic compounds on cardiomyocyte beating rate (Sirenko et al., 2013). The ultimate goal is therefore, to estimate cardiotoxicity earlier in the drug discovery process, which allows researchers to identify potentially toxic compounds and either eliminate or modify them to improve their safety profile. Eventually, these types of assays may detect ion channel blockers, adrenergic receptor antagonists, kinase inhibitors, anthracycline drugs, and other compounds capable of compromising the cell metabolic activity or viability or yet unidentified mechanisms of cardiac toxicity.

Recent results have demonstrated that cardiac cells are also responsive to other types of toxicities related to small molecule inhibitor activity (Chu et al., 2007). Indeed, hPSC-CMs responses are promptly amenable to evaluate additional or alternative toxic endpoints such as apoptosis, cell viability, ATP metabolism, and mitochondrial dysfunction through readily available test kits. Similarly, lethal arrhythmia is caused by the increase of an uncertain response by single cardiomyocytes (temporal aspect) (Jonsson et al., 2010) as a triggering agent and of cell-to-cell conductivity (spatial aspect) as an amplification/suppression agent (Antzelevitch, 2008; Bass et al., 2008). Increase of vague electrophysiological response produced by single cells could be the principal and essential origin to generate a lethal arrhythmia, and hence, the evaluation of fluctuation potential of single cells is crucial and should be the earliest index to predict lethal arrhythmia. However, the spatial location of cardiomyocytes should also be taken into consideration, such that lethal arrhythmia occurs in a tissue where each single cell presents a slightly different heterogeneity of responses, even under the same circumstances. Thus, cell-to-cell conduction is significant to estimate non-synchronized signal propagation in heart tissue.

A second origin of arrhythmia related to the community effects of cardiomyocytes leading to a temporal fluctuation of repolarization time of single cells after depolarization. This second origin could pose either enhancing or silencing roles for a lethal arrhythmia phenomenon. The heterogeneity of cardiomyocyte functional characteristics could augment the occurrence of lethal arrhythmia because of the divergent responses of neighboring cells from the first origin. In contrast, the community effect of cardiomyocytes also has the ability to suppress the occurrence of lethal arrhythmia by the enhancement of synchronization tendencies with suppression of fluctuation in cell groups (Kaneko et al., 2014). Kaneko and colleagues also described a promising way for predicting ventricular arrhythmia at the *in vitro* level by measuring the first origin of arrhythmia with temporal fluctuation of repolarization time on a single cell, and then measuring the second origin of spatial fluctuation of conductivity of neighboring cardiomyocytes by using spatially arranged hCMs. Kaneko and colleagues developed an on-chip cell network cultivation system, in which extracellular signals (field potentials: FP) of hCMs can be measured using a multi electrode array (MEA), and spatial arrangement control of cells can be performed using agarose microstructures designed on MEA chip. The drugs were

applied to the medium in the MEA chip, and the FPs were measured for 10 min at each drug's concentration. This cell-network-based *in vitro* assay has some potential advantages:

1. Using a set of standard hCMs prepared from human pluripotent stem cells of different race, gender, and from various diseased patients may provide an ideal testing panel platform.
2. To predict lethal arrhythmia evaluating the temporal fluctuation of ion channels kinetics and evaluating spatial cell-to-cell conduction using the on-chip cell network (Kaneko et al., 2014).

2.10 Hepatotoxicity

The liver exhibits diverse functions ranging from glycogen storage to the decomposition of heme from dead red blood cells. Of note, it also plays an essential role in clearing xenobiotics from the bloodstream, and therefore is highly susceptible to various types of toxic injury, which in some cases and through repeated exposure can lead to malignant transformation (Szkolnicka et al., 2013). Because the liver plays a major role in metabolism, mutations, or polymorphisms that affect metabolic activity can have profound effects on liver function and therefore susceptibility to injury (Szkolnicka et al., 2013). Drug safety is one of the primary concerns in drug development and liver toxicity is among the top organs for adverse drug reactions. In fact, drug-induced liver injury has been associated with over a third of acute liver failures in the United States and more than 1000 drugs are considered potentially toxic to the liver (Stine and Lewis, 2011). Indeed drug-induced liver injury is one of the major causes of drug candidate failure in preclinical and clinical testing (Corsini et al., 2012) and is also the most frequently cited reason for removal of approved drugs (Lee, 2003). While *in vivo* animal studies remain the standard for toxicity testing, they are time consuming and expensive, and more importantly, are rather poor predictors of human toxicity (Hartung and Daston, 2009). A number of *in vitro* models have been established for hepatotoxicity testing (Soldatow et al., 2013). For example, precision-cut liver slices (Elferink et al., 2011) contain all cell types of the liver in their natural architecture and have xenobiotic metabolism capacity. This model, however, is arguably not well suited for high-throughput studies. Immortalized cell lines, such as HepG2, and more recently HepaRG, are also widely employed (Gómez-Lechón et al., 2010). Cultures of primary (e.g., freshly isolated or cryopreserved) human, rodent, or canine hepatocytes have also been widely used for *in vitro* testing (Rodrigues et al., 2013). However, high inter-individual variability, limited availability, high cost, changes in cell morphology, and rapid de-differentiation of the hepatocyte phenotype in culture, particularly in the loss of cytochrome P450 (CYP) enzyme expression, are significant limitations. Human induced pluripotent stem cell (iPSC)-derived hepatocytes show great promise with respect to having a primary tissue-like phenotype, consistent and unlimited availability, and the potential to establish genotype-specific cells from different individuals (Anson et al., 2011). Therefore, there is still a clear need to improve current hepatocyte models, and to adopt new advances in experimental techniques to develop new models that will enable better prediction and understanding of the mechanisms causing drug-induced liver injury. With the rapidly advancing technology in stem cell research, it is envisioned that progress will be made in bridging this gap in toxicology research through the utilization of human embryonic stem cells (hESCs). Directed differentiation of hESCs to somatic cells with mature phenotypes in the laboratory could potentially provide a readily available source of metabolically competent cells such as mature hepatocytes with comparable functional status to freshly isolated hepatocytes for use in safety pharmacology and toxicology applications (Greenhough and Hay, 2012; Harris et al., 2013; Medine et al., 2013). More recently, pluripotent stem cells (PSCs) have been produced by the reprogramming of mature somatic cells, and are termed induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). This approach negates the controversies surrounding the use of embryonic tissue and potentially allows for in vitro modelling of normal and variant phenotypes for safety pharmacology and toxicology evaluations. As a result, iPSC-derived cell models are being adopted by the pharmaceutical industry

for preclinical toxicity studies (Kia et al., 2013; Rana et al., 2012; Reynolds et al., 2012). To realize the full potential of iPSC-derived cell models, it is necessary to develop predictive in vitro assays that can be performed in a high-throughput manner. Many different groups have attempted to ameliorate the differentiation of hESCs to HLCs (human liver cells) *in vitro* by mimicking the developmental pathway of the liver during embryogenesis. The aim is to derive mature hepatocytes from pluripotent hESCs using differentiation protocols encompassing the three main stages of hepatic development: definitive endoderm differentiation, hepatocyte progenitor specification, and hepatocyte maturation. To date, however, the perfect differentiation protocol has remained elusive. This is also compounded by the fact that currently there is no standardization of the methods used to characterize these HLCs and in assessing their differentiation potential. Comparisons between the various differentiation protocols could then be addressed, with the aim of developing one that is efficient, reproducible, and sufficiently robust for drug safety and toxicology screening. Human embryonic stem cells (hESCs) and induced PSCs (hiPSCs) may provide new opportunities for improving cell-based models owing to their renewable nature, plasticity, and isolation from a known genetic background. Although there are advantages in using hiPSCs compared with using hESCs as a starting cell source for differentiation into HLCs (Table 2.1), there are still limitations in efficient generation of hiPSCs. A new technique with the hope to improve the reprogramming efficiency works by the expression of defined microRNAs (miRNAs) to induce pluripotency in mature human somatic cells (Anokye-Danso et al., 2011). miRNAs play important post transcriptional regulatory roles in cellular and developmental events, they act as principal regulators by binding to a specific sequence motif of a target messenger RNA to induce their degradation or translational repression (Hobert, 2008). The use of specific miRNA clusters has been shown to induce pluripotency in human fibroblasts with a two order of magnitude increase in efficiency when compared with "classical" transcription factor-based cellular reprogramming (Anokye-Danso et al., 2011; Lin et al., 2011). Whilst these results demonstrated huge improvements in reprogramming efficiency and may allow for high throughput generation of hiPSCs, this technique still uses viral vectors for delivery of the miRNA cluster.

Table 2.1 *Advantage and Disadvantages for the use of hPS cells*

	Advantages	Disadvantages
hESC-derived hepatocyte-like cells (HLCs)	• More knowledge on the functional characteristics of hESC-derived HLCs multi-stage differentiation protocols developed.	• Limited genotypic variation with all the available hESC lines. • A viable human embryo is required and destroyed. The use of hESCs is subjected to ethical debate. • Limited number of cells isolated from each embryo.
hiPSC-derived hepatocyte-like cells (HLCs)	• Possible *in vitro* model of drug-induced liver injury for mechanistic studies. • Somatic cells are more readily available. Human embryos not required. • iPSC can be propagated indefinitely *in vitro* providing a renewable source of cells. • Potential to encapsulate the phenotypic variation of phase I and II enzymes present in the population by establishing a library of HLCs derived from different individuals representing the global and ethnic genotypic variation. • Prospective application in robust high throughput screening for drug-induced liver injury.	• Low reprogramming efficiency to hiPSCs from parental somatic cells. • Concerns with regards to the impact of genomic insertions from viral vectors used in the majority of methods for reprogramming parental cells. • iPSC may acquire genetic mutations during reprograming or *in vitro* culture.

Finally, it is now possible to produce functional HLC's from the desired human genotype. This holds great promise for modern medicine, not least the detection of human drug toxicity. The next step in the development of stem cell-derived toxicity assays will require an extensive validation period of retrospective studies. Drugs that have failed in animal studies or clinical trials as a result of safety issues will need to be screened with any new technologies developed, to establish whether toxicity could have been detected earlier on in the drug development pipeline using stem cell-derived HLCs. Taken alongside current approaches, stem cell-derived toxicity assays have the potential to improve the robustness of the drug development process and reduce the occurrence of late-stage attrition.

2.11 Epigenetic Profile

Susceptibility to disease in adulthood is associated with environmental factors during early development, and differential nutritional and occupational exposure. iPSC and SC can be used to study how the epigenetic profile may change during early development in response to environmental factors.

First, two concepts in the epigenetic profile have to be defined: (1) epigenetics and (2) toxicogenomics.

1. *Epigenetics* refers to the induction of stable changes in gene expression and chromatin organization that are independent of changes in the DNA sequence and can propagate through cell division (Herceg et al., 2013).
2. *Toxigenomics* refers to the molecular mechanisms that underlie adverse responses to a toxic agent, and measures the modifications in transcription levels of certain messenger RNA (mRNA). Such changes in transcript levels could lead to a modification in protein expression, and consequently the down- or upregulation of relevant pathways, which may interfere with normal cellular metabolism (Thomson et al., 2014).

Environmental factors can affect the epigenetic profile in primarily two ways: by changing the methylation pattern in the DNA, and/or by affecting the covalent binding of histone tails in the genomic DNA. Additionally, recent evidence has shown changes in microRNA expression may also affect the epigenetic profile. When this occurs in the embryo or fetus, these effects can lead to the development of disease in adulthood. Thus, understanding how the regulatory machinery in early development is affected by exposure to drugs, environmental pollutants, or chemical and biological stressors is essential for understanding adulthood disease (Thomson et al., 2014).

Residence adult stem cells in each tissue rely on specific transcription and epigenetic factors charged to maintain their own particular state of differentiation (Chen et al., 2012) and stem cells play an important role in regenerative process after tissue-damaging events as well (Iglesias-Bartolome and Gutkind, 2011). Exposure to chemicals may lead to changes in the epigenetic profile that may affect the capacity of self-renewal, differentiation, senescence, and homeostasis from the resident cells. Those changes could affect to the regenerative capacity of the tissue. Thus, the use of SCs or iPSCs as toxicological tools could obtain information about how changes in the epigenetic profile might affect homeostasis self-renewal, differentiation, and tissue senescence that could lead to disease development later on.

As was discussed previously, the stem cell population is particularly useful to assess processes such as cell self-renewal, differentiation, and tissue senescence (Gifford et al., 2013; Hemberger et al., 2009; Paige et al.; Raveh-Amit et al., 2013; Xie et al., 2014). Each of these processes contributes to the regenerative capacity of some tissues. Additionally, resident adult stem cells in each tissue rely on specific transcription, epigenetic factors and their epigenetic landscape to maintain their own particular state of differentiation (Boland et al., 2014; Chen et al., 2012). In addition, Iglesias-Bartolome and Gutkind found that stem cells play an important role in tissue homeostasis, during the regenerative process after tissue-damaging events (Iglesias-Bartolome and Gutkind, 2011).

Although the modulation of epigenetic targets on adult stem cell function can be modeled *in vitro*, at the moment it is not fully described and it is not feasible to develop assays that would comprehensively cover multiple adult tissues. Therefore, a more general screen based on human (or other animal models) embryonic or induced pluripotent stem cells (hESC/hiPSC) may serve as a surrogate for adult progenitor stem cells.

In conclusion, the use of iPSC or SC in the future could elucidate epigenetic profiles in individuals prior to the appearance of disease. Studying the patterns in the epigenetic profile of patients may allow for assessing their potential for future pathologies. Therefore, SC and iPSC could prove to be an effective tool for prophylaxis in the future, and hold tremendous potential for individualized health care.

2.12 Use of SC and iPSC in Drug Safety

Regenerative medicine is an important and growing field of research, which deals with replacing, engineering or re-generating tissues or organs using human cells in order to restore their normal function. A variant, which has been particularly fruitful, is *tissue engineering*, which refers to the use of organ-specific cells for seeding a scaffold *ex vivo* to treat disease.

In the last few years, stem cells and iPSCs have gained importance in regenerative medicine. Currently, various clinical trials make use of stem cell injection and report an improvement in the quality of life of patients, suggesting stem cell treatment is a new viable alternative to cure diseases. In particular, diseases such as heart stroke, diabetes mellitus type II, hematological deficiencies, and other malignancies, are currently being tested in clinical trials using a variety of stem populations, including embryonic (totipotent or pluripotent stem cells), mesenchymal or hematopoietic stem cells, or even these cells in combination. Although stem cells and iPSCs applicability is mainly in regenerative medicine, this is not their sole application.

Special attention has been directed to embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC's) and their applications. For example, ESCs and iPSCs have been proposed for assessing developmental toxicology, as well as an *in vitro* model for cardio- and hepatotoxicology assays. Other fields including medicine, pharmacology, tissue engineering, toxicology, and pharmaco-vigilance, could particularly benefit from the use of these stem cells (Wobus and Löser, 2011).

2.12.1 Potential Benefits of Stem Cell Use in Other Areas

Before much more progress is achieved, it is necessary to first develop new methods and strategies for stem cells culture and differentiation and manipulation in respect to their regulatory base. These new strategies and improvements should minimize the number of animals used, in time required for, and other costs associated with evaluating drugs, which are important limitations to the industry.

2.12.2 Methodologies

The use of stem cells may improve the methodologies used to evaluate and ensure biosafety. In veterinary medicine, for example, food ingredients may have differential effects across different species. Therefore, species-specific cell lines are required to evaluate their toxicological effects. For example, the evaluation of the biosafety of canine products is limited due to the absence of dog cell lines in the market. Consequently, mixture or drugs administered in veterinary medicine have to be assessed using alternative nonspecific cell lines. Thus, to improve *in vitro* screening, the use of canine bone marrow-derived mesenchymal stem cells (BMSC), could provide better information on canine ingredient safety (Ortega et al., 2015).

2.12.3 Economic Benefits of Stem Cell Use

The use of animals is essential to ensuring product safety. Manufacturers of drugs, vaccines, food additives, chemicals, water, air pollutants are studies, typically required to prove the safety of their products on animals. However, the relatively high costs and labor-intensive nature of drug discovery using rodent or other superior animals limits their usefulness as test organisms. Also, because experimentation requires a large number of animals, it can be quite costly.

Standardized protocols using iPSC or SC could reduce the number of animals required in experimentation and the time frame of drug discovery. These cells have therefore emerged as a powerful system for small molecules and for novel biological and therapeutic discoveries.

Finally, several animal models, focusing on different diseases have reported therapeutic effects of human ESC- and iPSC-derived progeny, (Keirstead et al., 2005; Laflamme et al., 2007; Lamba et al., 2009; Yang et al., 2008a, 2008b). These features together suggest that iPSC or SC are an excellent and emerging alternative for drug safety and risk assessment, to be applied in pharmacotoxicology, drug assessment, and biosafety.

2.13 Conclusions and Future Applications

There are optimistic expectations related to stem and iPSC applications in modern medicine, toxicology, and other areas. Strong efforts are being undertaken by pharmaceutical companies, universities, and government laboratories to uncover knowledge of iPSCs or SCs related to morphology, genetics, and physiology, to perform better toxicological assays to evaluate risk assessments and consequently significantly reduce time, animal use, and costs. SCs or iPSCs could increase safety studies and potentially could ensure human safety fulfilling regulatory requirements. In this regard, the concept of personalized medicine on stem cells isolated from patients for therapeutic reasons is particularly attractive. However, SC and iPSC applications are not limited to personalized medicine. The study of biosafety using specific stem cells or reprogrammed cells in industry, to evaluate pharmacodynamics and pharmacokinetic parameters, could bring important new data, and as noted previously, a reduction in the use of animals, a reduction in costs, and a considerable reduction in study time. Moreover, the inconsistent results derived from animal models related to physiology can be ameliorated with SCs or iPSCs as well. Thus, the extended knowledge of iPSC or SC can yield new data to ensure a safer and accurate study, changing modern toxicology and medicine, improving biosafety, and revolutionizing personalized medicine for those diseases difficult to treat by conventional means.

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3

Stem Cells: A Potential Source for High Throughput Screening in Toxicology

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3.1 Introduction

The field of science that evaluates the influence of external substances on living organisms is 'Toxicology'. From the 1950s, animals were used as experiments to predict the toxic levels of drugs and chemicals. Estimation of ocular toxicity, dermal toxicity, oral toxicity, immunotoxicity, genotoxicity, reproductive and developmental toxicity were done using animal models [1]. However, it was difficult to analyse the results from animal models and relate them to human systems. Additionally, the overwhelming dependency of pharmaceutical and biotech companies on safety screening has caused disastrous losses to companies, due to which the attrition rate of drugs into clinics still remains 40–50% [2]. Thus, there is immediate need for improvement in the toxicological assays to enhance the predictiveness of toxicity prior to animal testing. The Ames test was the first *in vitro* toxicology screening test to be designed and implemented. Additionally, acute cytotoxicity, chromosomal aberration and micronucleus test were designed and executed [3, 4]. However, these assays were low and inferior for long term as well as large scale screening.

Large scale screening of drugs and chemicals has been carried out by High Throughput Screening (HTS) assays, which were designed for screening genotoxicity, molecular toxicity, cellular metabolic activation processes, cytochrome P450 competition, embryotoxicity and endocrine disruption [5]. However, standardization, validation and recommendation from toxicologists was still a challenging task. Registration, Evaluation, Authorization and restriction of chemicals (REACH), a European community on regulation for

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safe use of drugs and chemicals was established, which demanded HTS assays help to classify the compounds based on the toxicity levels before entering animal testing [6–8]. Several strategies were implemented to measure the extent of toxicity at genome level by screening toxicity at nucleotide level, chromosomal level and nucleus levels [9]. Major strategies focused on the sources of cells that can represent human physiological conditions. *In vitro* testing platforms have been improvised for reducing and replacing the animal usage [10]. Primary cells and immortalized cells are used for testing and screening of chemicals and drugs. However, due to the irrelevance/lack of sufficient cell numbers and loss in functional property in long term experiments have demanded an alternative cell source for toxicity studies and HTS [1]. Stem cells with potential features have gained increasing scientific interest in biomedical industries. The undifferentiated state and self-renewal properties have allowed stem cell approaches in regenerative medicine to investigate an array of diseases. Predictive toxicology as well as drug discovery programs and other platforms that rely on differentiation capacity of stem cells to develop into 3D engineered tissue equivalents for toxicity assessment are also representing the new horizon of alternative models replacing animal models in testing and screening of drugs [11, 12]. In this chapter, we focus on the importance of stem cells in HTS assays for evaluation of toxicity and testing of chemicals/drugs for safety and efficacy.

3.2 Stem Cells

Stem cells show the remarkable property of self-renewal and the capability to divide an unlimited number of times as well as differentiate into various cell types. A stem cell upon division has the potential to become either a stem cell or a progenitor of any specific cell type. Stem cell renewal is a regular process in some organs like gut and bone marrow, which helps to replenish the old worn out cells; on the other hand, stem cells can divide under specific conditions in organs like the heart, pancreas and so on. Stem cells can be considered as the cell reservoir system to repair, regenerate and replace the damaged tissue/organ of the body tissues/organs [13]. Stem cells reflect the potential immune response against diseases, repair system, cell therapy and tissue regeneration; these salient features have meant that stem cells gained a major frontier in the scientific world of biology and medicine.

Stem cells have been classified into many cell types that differ in the degree of differentiation and self-renewal ability. Gametes (eggs and sperms) are stem cells that are able to transform themselves into a whole body consisting tissues after fertilizing. Stem cells derived from blastocyst stage of embryo development are called embryonic stem cells, which show the ability to differentiate in to any cell types. Adult stem cells are found in tissues or organs and have partial differentiation ability. Based on their degree of plasticity, versatility and tissue origin, stem cells have been classified into diverse specialized cell types. A brief description of stem cell diversity and classification has been listed in Table 3.1. The advent of technology and applications of stem cells in medical fields such as cancer, leukaemia and blood/bone cancer has showed promising success. Thus, stem cells are proving to be the most potential, multi-purpose and cost effective cell source among those existing.

3.2.1 Embryonic Stem Cells (ESCs)

In 1981, culturing of mouse embryos in uterus and isolation of embryonic stem cells from embryos was demonstrated for the first time [14]. In the same year, *in vitro* culturing of embryos and embryonic stem cell isolation from the blastula stage of embryos was performed [15]. In the late 1990s, James Thomson and team reported the first successful isolation and propagation of human embryonic stem cells (ESCs) under *in vitro* conditions [16]. Mammalian embryos have 50–200 cells during the blastocyst stage, 4–5 days after fertilization in humans. Inner cell mass of blastocyst consists of ESCs, from where they are isolated and cultured for

Table 3.1 *A brief description of various stem cells is discussed*

Cell Type	Characteristics
Embryonic stem cells	Pluripotent in nature. Derived during the early embryo stage from inner cell mass of blastocyst.
Adult stem cells	Stem cells found from the specific tissue/organ type, e.g. epidermal stem cells (skin), neural stem cells (brain), etc.
Cancer stem cells	Found at cancer/tumour site with abnormal dividing capacity.
Induced pluripotent stem cells	Somatic cells that are synthetically stimulated to 'induce' pluripotency.
Totipotent cells	Cells having ability to give rise to all cell types of an organism and develop into functional organism.
Pluripotent cells	Cells that can give rise to all tissues/organs but not able to form whole organism.
Multipotent cells	Cells that can give rise to only limited number of cell types, e.g. hematopoietic stem cells.
Unipotent cells	Precursor cells.

further propagation. Embryonic stem cells (ESCs) show remarkable properties such as the ability to differentiate into all cell types of three germ layers, capable of self-renewing for an unlimited number of times, under defined media conditions [17].

Identification of several proteins have marked the 'stemness' phenotype of ESCs through expression of specific genes such as Nanog, Oct4, Alkaline Phosphatase/ALPL, E-cadherin, SOX2, SSEA-1, TRA-1–60 and many others have been used for evidencing the pluripotency stage of ESCs. Landmark genes are useful in designing protocols of ESC-differentiation into specific cell types. Thus ESCs under *in vitro* conditions can offer access to unlimited cell source for differentiation protocols. ESCs could be used for regenerative medicine, studying genetic disease, signalling pathways and also for *in vitro* toxicology studies [18]. Discovery of human ESCs took the lead in understanding human embryology and also in differentiation of multipotent progenitors for neural, cardiovascular and hematopoietic lineages, as well as for differentiation protocols of various cell types for efficient generation of cardiomyocytes, dopamine neurons, immature pancreatic β-cells and so on [19]. Although, ESCs show up the advantageous features, it is challenging to standardize and optimize efficient differentiation protocols to conserve functional features on the *in vitro* and pre-clinical levels. For example, designed protocols should not only focus on efficiency but also on regulation of functional features too, namely albumin secretion, indocyanine green uptake and release, glycogen storage and p450 metabolism [20]. However, due to difficulty in controlling the efficiency of a cell's function and ethical concerns, implementation of human ESCs and human ESC-derived cells are still in the early stages of reaching clinics for human use.

The first step towards human ESC applications for human use was recently updated by Food and Drug Administration (FDA). The FDA approved the world's first application of human ESCs in human clinical trials and this represents an exponential development in technology for improving human life, Phase I clinical trials for spinal-cord treatment were done for transplanting oligodendrocytes derived from human ESCs [21]. Along with the rapid development of biomedical application of ESCs, ethical concerns and safety concerns are issues to be worried about. More importantly, side effects of ESCs in their possibility of forming tumours, such as teratoma [15]. Strategy to enhance the safe application of ESCs in clinical trials is to use the differentiated ESCs in to specific progenies, additional precautions should be taken by (Fluorescence Assisted Cell Sorting) FACS sorting the cells for further purification. ESCs are inherently much safer than induced pluripotent stem cells (iPSCs), since ESCs are not induced at gene level to modify genes such as c-Myc, which is linked to cancer [22]. However, strategies have been implemented to improve the safe elimination of Myc expression and to retain the 'stemness'.

During the early 1990s, scientists commenced research on ESCs for *in vitro* toxicity prediction [23]. European Centre for Validation of Alternative Methods (ECVAM) has developed advanced versions of *in vitro* toxicity tests for evaluation of sensitization, photo-toxicity and embryo-toxicity [24, 25]. Strategies were implemented to consider ESCs for toxicity studies. Stem Cell Research & Toxicology (SCR & TOX), a European consortium, aimed to make use of human ESCs to establish assays for screening toxicity of materials, pharmaceuticals and cosmetics [26, 27]. ECVAM validated the Embryo-toxicity Stem cell Test (EST) for predicting embryo toxic compounds. The significance of EST was confirmed with screening and categorization of certain chemicals based on their extent of toxicity as well as *in vivo* consequences on animal and/or humans [28]. Since then, EST has been considered as the standard test for embryo-toxicity assessment [29, 30]. Additionally, ESCs have proven to be useful in the field of dental science in creating the awareness of toxic effects stimulated by dental/oral materials [31]. Various reports have been documented highlighting the importance of human ESC-derived cells as an alternative platform for toxicology screening.

3.2.2 Foetal Stem Cells

Primitive cell types are found in the organs of foetus and called foetal stem cells. Foetal blood, bone marrow and foetal tissues has abundant number of foetal stem cells. Hematopoietic stem cells are one among the foetal stem cells found in the placenta and umbilical cord. Additionally, liver, kidney, neural stem cells and pancreatic islets progenitors can be also isolated from foetal tissues/organs [32]. Hematopoietic stem cells have shown to be an effective treatment in blood diseases such as leukaemia, anaemia and various applications in biomedical research. However, for long term investigations, storage of foetal stem cells was challenging until advancements in storing and banking cells was implemented by cryopreservation. Cryopreserved cells can be utilized for further studies for liver toxicity, developmental toxicity, leukaemia, anaemia and other medical applications in the future [33]. Foetal stem cells have been used as a platform for studying and understanding most devastating diseases. Foetal stem cells have shown promising source also for investigating developmental toxicity. Foetal stem cells are more studied in female populations, since toxicity can hinder female reproduction and foetal development, thus deserves special attention [34, 35]. In addition to this, toxic effects on foetal stem cells might hamper development, organogenesis, morphology, foetal reproductive function and impairment in the foetus. However, investigations on toxic effects on foetal stem cells carried out on different animal models should be conducted for multiple generations to confirm the genotoxic levels. Since it is unrealistic to rely on animal models due to differences in endocrine systems of human and animals, results gained from such investigations are unacceptable for human studies [36–38]. Implementation of foetal stem cells for toxicity studies and drug discovery projects as well as to scale up via high throughput screening will be an efficient approach to commercialize technology.

3.2.3 Adult Stem Cells

Adult stem cells are isolated from mature tissues/organs. Adult stem cells are undifferentiated cells found all over the body, which helps in maintaining tissue homeostasis by regenerating and replacing damaged cells/tissues. Adult stem cells are self-renewing and multipotent in nature. Adult stem cells undergo symmetric division (gives to identical daughter stem cells) and asymmetric division (gives to one stem cell and other progenitor cell with limited self-renewal capability). During differentiation, adult stem cells undergo various signalling pathways, such as notch pathway, Sonic HedgeHog signalling and Wnt pathways [39]. Development stage of adult stem cells restricts their potential in comparison to embryonic and foetal stem cells [40]. Adult stem cells are lineage restricted and tissue-oriented cells, such as adipose-derived stem cells, bone marrow stem cells, neural stem cells and so on. Adult stem cells show vital roles in the tissue repair and regeneration of their respective loci [41]. However, isolation of adult stem cells doesn't involve destruction of the embryo

as ESCs do. Thus, implementing an adult stem cell is not as controversial as embryonic stem cells. Adult stem cells can also be isolated from recipient (autograft) to overcome tissue rejection issues.

Bone marrow stem cells (BMSCs), hepatic stem cells, cardiac stem cells as well as skin stem cells are the most frequently used adult stem cells for toxicity studies and drug discovery. Two precursor cell populations are found in adult BMSCs, hematopoietic stem cells (HSCs) and marrow stromal cells (MSCs) [42]. Multipotent HSCs are found in circulating blood and umbilical cord blood (UCB) and regulate the production of blood cells throughout life. MSCs are also multipotent in nature. MSCs can be isolated from adipose tissue, placenta and amniotic fluid. MSCs have potential to differentiate themselves into osteocytes, adipocytes, chondrocytes, smooth muscle cells and hematopoietic supportive stroma [43]. Human HSCs have been characterized with staining of Lin, CD34, CD38, CD43, CD45RO, CD45RA, CD90 and many more antibodies [44]. In addition to surface markers, metabolic markers such as rhodamine123, Haoechst3342, Pyronin-Y have been used to screen HSCs. For MSC characterization, CD106, CD105, CD73, CD29, CD44 are commonly used [45]. BMSCs have issues with clinical applications since there is the concern about transplantation due to immune rejection. However, chemotherapy and radiation therapy have overcome the existing clinical problems of transplantation of bone marrow and peripheral blood stem cell transplantations. Novel and promising technologies of tissue engineering, regenerative medicine and cell therapy are implementing the application of isolated HSCs and MSCs. The growing field of molecular medicine has highlighted important need and application of cell sources/bio-specimens that simulate the human *in vivo* niche and provide an efficient platform for researchers to make predictive assessments of new compounds for safety and efficacy [46, 47]. In the scenario of drug induced toxicity, bone marrow is the prime target to be affected. Thus, bone marrow and its derived cells have become an important tool to contribute for toxicity predictive platforms in the field toxicology and drug discovery [48, 49]. However, the traumatic procedure of BM extraction and limited amount of cells brings up challenges and allows us to explore other cell sources as well as isolation procedures.

3.2.4 Adult Stem Cells in Other Tissues

Adult stem cells are known to be present in the mammalian tissues of adult bodies and play a role in the repair and regeneration of tissues. Loss of functional efficiency of adult stem cells highlights ageing [50]. Adult stem cells that are known for their vital functions have been discussed. Neural Stem Cells are one of the most sensitive cells of the body. Neural stem cells vary from species to species in representing the development pattern of brain. Development of mammalian brain highlights seven major areas: olfactory bulb, ependymal (ventricular) zone of lateral ventricles, sub-ventricular zone, hippocampus, spinal cord, cerebellum and cerebral cortex. During normal development of the brain, along with proliferation and differentiation of neural stem cells, even genetically programmed events also hold responsible reasons. Neural stem cells have been proved to be a potential platform for studying developmental neurotoxicity as well as for investigating long lasting neuronal impairments [51–53]. In addition, the previously mentioned adult stem cells, there are some challenging tasks to distinguish between adult and embryonic category, cells such as endothelial progenitor cells that cover the inner lining of blood vessels have proved to be originated from the hemangioblasts of the mesoderm. The process of blood vessel formation in an embryo is called vasculogenesis. However, in adults, the process of blood vessel formation from pre-existing blood vessels is termed angiogenesis [54]. Endothelial cells have proven to be an acceptable and reliable model for tumour modelling, drug screening, anti-angiogeneic studies and drug discovery applications.

Biomedical and tissue engineering research have gained high levels of interest in studying regeneration capabilities of adult hepatocytes, liver progenitors and stem cells. During liver tissue homeostasis, adult hepatocytes undergo a reduced rate of cell division through which matured hepatic cells go through the cell cycle as well as regaining damaged tissue [55, 56]. There is potential to use isolated adult hepatocytes for

suitable treatment of liver diseases in both animal and human livers. Liver transplantation is one of major solutions of critical hepatic diseases. Liver replacement treatments are generally restricted by number of donors, immune reactions and tissue/organ rejection [57]. Hepatic stem cells have been shown to play an important role in understanding the liver development, regeneration, investigation of liver toxicity and also aiming towards reliable transplantation. Various *in vitro* systems have been developed to investigate the adverse effects of drugs and chemicals on liver. Extensive use of liver slices, perfused liver setup, immortalized cell lines and primary cells are involved in existing platforms [58]. However, due to several limitations, an alternative source of hepatic cells is needed for predicting toxicity. Advancements in the field of *in vitro* hepatotoxicity, including three-dimensional tissue engineered equivalents, artificial livers, co-cultures systems and differentiation protocols of stem cells into hepatic lineage-like cells, have attempted to provide a more physiological environment for cultured liver cells by incorporating fluid flow, micro-circulation and other forms of organotypic microenvironments [59]. Stem cells, both embryonic- and adult tissue-derived, may provide a limitless supply of hepatocytes to improve reproducibility and enable testing of the patient-specific toxicity [60]. In addition, an unlimited source of stem cells provides a platform to scale up the technology via high throughput screening for drug testing and discovery applications.

Due to excessive use of animal models by research institutes, academies have created awareness to reduce animal usage and simultaneously encourage alternative research tools that can solve biological equations in a far more promising way. Stem cell technologies are one among the alternative approaches. Salient features of stem cells provide platforms to generate different cell types and opens doors to designing possible stem cell-based platforms, protocols for large scale productions, high throughput screening and mass production.

3.3 High Throughput Screening (HTS)

In the last two decades, High-Throughput Screening (HTS) has obtained popularity in teh drug discovery field. HTS is an approach to scaling up the screening process of drug discovery, drug testing, toxicity screening of libraries of chemicals and drugs. HTS includes various steps like identification of targets, reagent preparation, compound development, assay development and high-throughput screening [61]. HTS has gained popularity not only in industry but also in academic research institutes. Productivity challenges faced by drug discovery industries have created financial pressures, a rise in the ratio of cost to commercialization, short-term profits and negative effects on the scientific field; the post-genome era heralded a significant increase in therapeutic interests for screening small molecules. Along with these, regulatory authorities have made strict regulations for the safety of drugs and pharmaceuticals, which led to an increase in substantial stress in research and development of pharmaceutical industries. Additionally, drug discovery strategies rely on mass screening the libraries of biochemical and biomedical drugs against various extra and intra-cellular molecular targets to match the desired functionality of compounds [62]. These enormous amount of evidence highlights the potential advantages and importance of HTS in toxicology and drug discovery. Over the last two decades, the concept of combinatorial and multi-parallel chemical synthesis, automation techniques for the isolation of natural products and availability of large compound libraries have diversified the compound collections of biomedical and pharmaceutical companies [63, 64]. In parallel, human genome sequencing, as well as sequencing the genomes of various pathogens such as viruses, bacteria and microbes showing the potential impact of high-throughput screening in understanding molecular functions to pursue drug discovery, has come into existence [65]. There has been a transformation of screening procedures from using test tubes and cuvettes for measuring high-density, low-volume assay and screening assays. This transformation has showed rapid changes in screening, identification and validation of biomolecular as well as biochemical targets [66]. HTS has provided increased reliability and reduced personnel workload with great facilitation of

data analysis tools and standardizing assays, thus making biomolecular and biochemical screening faster and more efficient than before. HTS has become a rapidly growing field by rewarding the great demands in productivity of drug discovery and development with highly efficient quality drugs. HTS gains new opportunities for the biomedical field in predictive toxicology and drug discovery [67]. During the early and mid-1990s, the evolution of HTS advancement showed the importance of microplates with 96 wells per plate that became the standard plate format being used for chemical and drug screening in major pharmaceutical and biotech companies. However, over the past decade there was need for improvement in plate format to scale up screening procedures and to meet the rate of production of drugs. Introduction of microplates with 384 wells per plate can accommodate four times more samples than 96-well microplates, thus reducing working volume range [68]. For all the major assays based on cell biology, biochemical aspects were adapted using the 384-well microplate without any compensation made by the results [69]. Several companies were able to miniaturize and inculcated 1536- and 3456-well microplates. Irrespective of variations in results, these plate formats face hurdles in managing minute working areas and volume, thus high expertise is needed [70]. Scaling up features as well as the cost effective approach of HTS show numerous applications in toxicology, drug discovery, predictive biology and pharmaceutical industries.

3.3.1 Current Strategies and Types of High Throughput Screening

Emerging trends of miniaturization and automation in bioanalytical techniques have gained demand in the pharmaceutical industries as well as in academic research institutes. Reducing the costs with increase in the ease of handling with minimal space consumption is the advantageous part. The process of automation in HTS done with multiple layered computers, several operating systems, a central robot and a scheduling software. The central robot helps to pick and place the microplates around the platform. Runtime varies from assay type during which plates are processed. Around 400–1000 microplates can be processed during a scheduled runtime. Loading of reagents in microplates and transfer of microplates to processor can be regulated by robotic programs. Thus, robotic pick and processing of microplates allows a one-step assay for screening library of chemicals and drugs [71]. However, it should be noted there has been a significant decrease in readout timings, actual screening process and large degree of automation as well as fast readout technologies that have compensated with overall turnaround time of the project. Thus, validation, standardization, development, implementation, adaptation, data analysis and interpretation as well as follow up of secondary assays have become major time consuming steps. Additionally, HTS efforts have direct and indirect effects on costs of screening programs since instruments, reagents and other consumables are needed for shorter and longer time periods. The major cost driver for screening is not only technical instruments, even typical reagents like biological test samples, antibodies, proteins, cells substrates and so on, as well as consumables such as tips, microplates, vials and so on are also valid reasons for increasing cost. Very sensitive assays, expensive assays, assays with highly readout technologies (e.g. time-resolved fluorescence resonance energy transfer [TR-FRET]) can be uneconomical in terms of cost effective assays [69]. The quality of process is also an important criterion to be considered for drug discovery using HTS technology. Screening of large data sets should be done properly, since only high statistical quality data sets can be approved for data analysis. For appropriate statistically significant and reliable results, the number of false positives and number of false negatives should be considered. However, overall quality of assays should not only be expressed due to statistical significance but also by the biochemical or biological sensitivity of a specific assay setup for detecting compounds with inhibitors or weak affinity molecules [72, 73]. During optimization and standardization, one should consider the interdigitated link between time, cost and quality of HTS assays. HTS assays are broadly categorized into two major assay types: *in vitro* bio-chemical assays and cell-based assays. There is further classification of biochemical assays into molecular binding (affinity) and *in vitro* functional assays (e.g. enzymatic reactions).

3.3.2 *In Vitro* Biochemical Assays

In advanced HTS, *in vitro* biochemical assays play a vital role in dealing with extensive targets against various enzymes, receptors, ligands, proteins and so on. *In vitro* biochemical assays are also useful in understanding interactions between ligand-protein, protein-protein and receptor-protein. This approach of HTS assays facilitates understanding molecular mechanisms, delineating metabolic pathways and signalling mechanisms as well as various biological, biochemical and genetics studies that are involved in diseases [74, 75]. Optimization of the approach offers the advantage of specific target-drug interactions and clear structure-activity relationships (SAR). In drug discovery, molecular binding interactions to understand biological processes and enzymatic events are the key elements of HTS assays. Various enzymes were being implemented in therapeutic processes to read in HTS for miniaturizing the experiments using microplates and automated screening of compounds and mutant enzymes [76].

3.3.2.1 Fluorescent Based Assays

Commonly used HTS detection methods are fluorescence based. Sensitive readouts provide homogenous results even at each single molecule level and also allows us to miniaturize the process. Fluorescent based detection involves methods such as fluorescent intensity (FI), FP, fluorescent resonance energy transfer (FRET), TR-FRET and also single molecule detection techniques like fluorescence intensity lifetime and fluorescent correlation spectroscopy (FCS) [77, 78]. Assays measure the change in intensity of fluorescence using fluorogenic substrates/internally quenched substrates. Detection of fluorescence signal from enzymatic reaction or quenching secretion helps in analysis of assays. Hydrolase detection assays such as protease assays are the common types of assays in which fluorescent intensity is mainly used as detector [79]. Measurement of FRET is done based on the intensity of quenching donor fluorescence by an acceptor or by emission of sensitive fluorescence of an acceptor. FRET assays helps in assessment of different types of target such as proteases, kinases [80]. The principle of TR-FRET assays is similar to FRET assays, including the basis of fluorescent resonance energy transfer between the donor and acceptor molecules. TR-FRET assays have the advantage of long-term fluorescence properties and allows a relatively large barrier compared to FRET moiety pairs for energy transfers [81]. FRET and TR-FRET assays are widely used in detecting cell metabolites [82]. Some assays are designed to determine molecular interactions in solution. Based on the excitation of polarized light by the fluorescent molecules, the degree of emitted light retained is directly proportional to molecule's rotational relaxation time. In a solution, small molecules rotate faster giving a lower polarization value whereas large molecules rotate slower and give higher polarization value. Polarization and anisotropy are used for detecting molecular interactions. During binding or enzymatic reactions, change in mass of fluorescent labelled molecules can be detected by *fluorescence polarization assays* [83]. Various fluorescence based assays have been designed for screening applications, namely *fluorescent correlation spectroscopy* [84], *one- and two-dimensional Fluorescence Intensity Distribution Analysis* (FIDA) [85] and *Fluorescence Micro-volume Assay Technology* (FMAT) [86]. Additionally, Luminex assays and Heterogeneous fluorescent assays have also been inculcated with HTS technology. The principle of Luminex and Heterogeneous assays is based on the mechanisms of flow cytometry and ELISA, respectively [87, 88].

3.3.2.2 Luminescence-Based Assays

Luminescence is highly sensitive and shows negligible background signals compared to fluorescence technologies. Luminescence includes bioluminescence, chemiluminescence and electrochemiluminescence. Similar to TR-FRET assays, another versatile and nonseparation screening technology called the AlphaScreen

Assay was developed based on the luminescence principle. More particularly, chemiluminescence of beads is measured when a donor bead with photosensitizer absorbs light and converts oxygen to its singlet state at 680 nm. Acceptor beads containing thioxene derivatives react with singlet oxygen to release chemluminescence signals at 370 nm. Emission of light at 520–620 nm was shown by excitation of fluorophores of the beads. A significant feature placing AlphaScreen assays over the TR-FRET assay format is that the amplification capability of signals is huge and brings with it a more sensitive assay [89]. However, variations in results is greater in AlphaScreen assays than TR-FRET assays. The AlphaScreen assay format can be used for *in vitro* screening, binding, enzymatic assays, probe detection in cell lysates and also in specific proteins [90]. Electrochemiluminescence (ECL) uses bead-based technology in which ruthenium acts as a chelating agent that conjugates with an antibody for tracing the signals. Upon reacting with tripropylamine (TPA) luminescence signals are emitted after applying low voltage. Alignment of beads is done by magnetism; these assays are used only in HTS diagnostic bio-assays. Several luminescence based assays are coming up with novel concepts including well known enzymes like horse peroxidase and ELISA assays. Novel approaches have been implemented for advanced utilization of luminescence in biomedical research. Some new assays like Caspase Glo, Kinase Glo for detection of ATP, cytochrome P450s have been designed [91, 92].

3.3.2.3 Colorimetric and Chromogenic Assays

Colometric detection is less sensitive than the fluorimetric approach. Chromogenic assays are based on the readout paths of light passed through a liquid sample, this mechanism is based on the Beer–Lambert Law. Thus, miniaturization of this technique cannot be taken to a lower volume of liquid sample. Screening artefacts were also encountered from Colometric assays, since coloured compounds present in the compound libraries can interfere the detection efficiency and accuracy [93, 94]. For the detection of a variety of enzymatic reactions, coupled assays were designed. Coupled assays are based on the reactions of enzymatic reactions with reactive chemicals or products or substrates of a particular reaction initiated by a second enzyme. Coupled assays are advantageous in monitoring activity of enzyme libraries by using a single and common analyte. HTS assays for *in vitro* analysis has increased the applications of enzyme complementation [95, 96].

3.3.2.4 Mass Spectroscopy (MS) Based Detection Assays

Mass spectroscopy (MS) is new tool for detecting analytes. MS is usually combined with liquid chromatography (LC) for developing a label free method for detecting analytes, substrates, products and so on. Analysing the ratio of mass to charge (m/z) has provide to be a successful approach in HTS assays using MS/LC setups. Screening of metabolic enzymes in large scale at industrial sites have been conducted. Efficiency was improved by multiplexing the LC columns by running them in two parallel screens; meaning more compounds in a short span of time. Over 6–8 weeks' duration, nearly a million compounds can be screened using the MS/LC approach in HTS [97, 98]. Thus, MS/LC in HTS shows a very reliable and promising platform for large scale screening in near future.

3.3.2.5 Chromatography-Based Assays

Separation of bound and unbound molecules by use of the chromatography technique is one of simplest methods to detect unbound ligands. This method was initially used for detecting small molecules bound to serum albumin [99]. The chromatography approach has been improvised by including size exclusion chromatography (SEC) for large scale screening, which can be used for HTS to separate bound and unbound

molecules from the pool of small molecules. Later, bound compounds were subjected to LC/MS for analysing based on their mass. The approach has been inculcated with SEC and automated to increase the throughput process. Alternative to SEC, a new modified approach, size exclusion ultrafiltration, in which the protein-ligand complex is trapped into the filters preventing it from flowing through, has implemented [100]. However, the SEC method is more efficient in single sample processing than multiple parallel sample processing.

3.3.2.6 Immobilization and Label-Free Detection Assays

Immobilization and label-free detection systems are the techniques for detection of a target that is either bound with a specific antibody or for detecting the surface changes of an immobilized target. ELISA and ELISA-based assays can be categorized into immobilized detection methods, in which there is requirement of selectively labelled binding molecules like antibodies, oligonucleotides and so on for a desired protein [101]. Among the many surface treated or surface modification detection systems, Surface Plasmon Resonance (SPR) technology is one of the best change in surface detection system to understand interactions between molecules with an immobilized target. In recent years, label-free, surface change detection and immobilization systems have gained a demand in cell culture based assays, *in vitro* systems and also in HTS systems to scale up and improve the efficiency of screening. With advancing technology, s few systems like Resonance Acoustic Profiling (RAP) and Wave-length interrogated optical Sensing (WIOS) have come up. Strategies like change in surface chemistry and size of ligands interacting to immobilized substrate have already been implemented into a 384-wells plate format for HTS applications [102]. Frontal Affinity Chromatography (FAC) is also an example of an immobilized detecting system. FAC combined with MS (FAC-MS) can be used in monitoring the effect of small molecules bound to proteins. In this method, a chromatography column matrix consists of an immobilized protein or target of interest. Ligands or small molecules are flushed through the column in presence of running buffer. Compounds having affinity will bind to the immobilized protein and tend to be retained on the column, thus delaying the elution time. The longer the elution time, the higher the affinity and higher binding. Eluted compounds can be analysed by MS [103, 104]. This approach is useful to screen mixtures of two or more compounds and also helps in separation of compounds. However, this technique has some flaws, such as immobilization of protein should be done in sufficient amounts because once the protein molecule interacts with the target molecule, the protein won't be able to bind with another molecule.

3.3.3 Cell-Based Assays

Since the last decade, the application of cell culture platforms of screening drugs, pharmaceuticals has increased in drug discovery by HTS assays. Screening of natural products and plant extracts for antimicrobial activity have been done using cell-based assays [105]. Additionally, cell-based assays have been used to monitor cellular mechanisms in the presence of specific molecules and the advent of molecular biology techniques has also increased the importance of cell-based assays, which can be used in drug discovery, toxicity testing and HTS. Cell-based assays have also played a vital role in understanding biochemical mechanisms of target based-chemicals to assess the toxicity and compatibility in cellular micro-environments. Thus, assays can also be used to tune chemical molecules to match biological relevance and quality of analysis can also be improved. Screening and drug discovery can be performed by cell-based assays such as reporter gene assays, secondary messenger assays, cell-based ELISA and pathway screening assays for extensive use of cell sources. Several assays have also been designed to evaluate the nature, locality and reaction of cell organelles and cell secretions by using binding property specific proteins as well as peptides or indirect assays to monitor nonspecific cellular reactions.

3.3.3.1 Reporter Gene Assays

Reporter gene assays are designed specially to track down the changes at gene level in both intra and extra cellular compartment. Reporter gene assays shows specificity among enzymes or fluorescent protein expression levels, which links to the expression of RNA or promoter that regulates the gene of interest. The same strategy has been used in bacterial systems for selecting antibiotic resistant reporter systems such as β-galactosidase or chloramphenicol acetyl transferase. Reporter gene systems are also useful in drug discovery studies as well as in understanding mechanisms involved in chemical compound reactions [106]. In spite of improvements in technology, reporter gene assays couldn't satisfy bacterial systems for long term use in HTS. However, mammalian cell systems have been implemented these assays to improvise HTS technology. Several systems have been developed based on the Reporter Gene assays that are used in HTS technology, especially with mammalian cells. The β-Lactamase (*bla*), which is available for mammalian cellular assays. CCF2-AM dye (fluorescent based dye) is commonly used in the β-Lactamase system. Dye is based on a set of an acetate ester group that actually reacts with intracellular esterase and accumulates in the intracellular spaces. Screening of effectors, peptides which are associated with translation and translocation process can be done using the *Secreted Alkaline Phosphatase* (SEAP) assay. The assay is basically the chemiluminescence of intercellular located proteins that allows monitoring of gene expression [107–109]. The luciferase system is one of the most commonly used reporter gene assays that shows sensitive signals, a large range of samples and ability to use photon multiplier tube (PMT) readers to detect biological activity in cellular systems. Sequential detection of two different promoters can be done. Normalization, correction of errors and standardization can be done using a control gene. Luciferase activity can be used in detecting the close protein-protein interactions using bioluminescence resonance energy transfer (BRET) [110, 111]. Enzyme complementation assays were developed to monitor intracellular locations of specific proteins or receptors. Translocation events were used as landmarks for assessment of enzyme activity in cellular assays. For more sensitive assays, green fluorescent protein (GFP) was used for fluorescent signals to detect protein-protein interactions, whose mechanism is similar to the Luciferase assay [112, 113]. Among several fluorescent or bioluminescence proteins, Green Fluorescent Protein (GFP) is a widely used fluorescent tracking protein for cellular imaging, especially in high content screening. In BRET assays, GFP and GFP derivatives fluorescent proteins are also used for imaging and high throughput screening [114]. Improvements have been made to standardize ELISA assays; horse redox peroxidase enzymes were used for chemiluminescence approaches to implement the cell-based western blot assay, also called CytoBlot assays [114, 115]. These standard assays are useful in scaling up the screening of analytes from whole cells.

3.3.3.2 Cell-Based Label Free Readouts

Screening technologies have enabled detailed pharmacological evaluation of cells, surface receptors and even nuclear receptors. Advanced systems like the CellKey system work based on electro-impedance using cellular dielectric spectroscopy (CDS) or Real Time Cell Electronic Sensing (RT-CES). Non-invasive electroimpedance can detect the presence, absence or changes in properties cells or molecules affected by electronic and ionic properties of sensors present on the cell surface, thus allowing real time label free-kinase measurement of several receptors. Similarly, evanescent wave technology was used in detecting cell changes in micro-titre plates [116, 117]. Cells grown in individual wells with sensor-containing micro-titre plates have not showed any changes in their biological activities. These assays were implemented in 96- and 384-well plate formats to HTS of various chemicals and drugs. Simplest label free cell-based assays are to monitoring growth cycles, population doubling and proliferation rate of cells [118]. Majority of screening assays use inhibitory effect of cells to access the antimicrobial as well as anticancer activity [119]. Improvement in the conventional assays were done to monitor multi-target screening and to develop similar systems that use

several model organisms, co-culture systems for identifying mechanisms behind interaction of compounds with bacterial system and pathogenesis of bacterial systems, thus mimicking *in vivo* milieu [120, 121]. In addition to cell-based label free assays, few of the *Ion channel* assays are being developed. *Ion channel* assays are based on the principle of conventional patch clamp experiments and coupled with a fluorescence plate reader. *Ion channel* assays can detect changes in calcium concentration, membrane potential. The latest breakthroughs such as automated electrophysiology, *IonWorks Quattro* and others have improved the *ion channel research field* [122, 123]. However, this type of assay requires highly skilled man power and other challenges in interpreting the true signals by masking false positive ones. Thus, the *ion channel* assay system is still in its infancy and has yet to create an impact in HTS technology.

3.4 Need for a Stem Cell Approach in High Throughput Toxicity Studies

A decade ago, the importance of cell-based assays increased in the field of drug discovery, toxicity testing and screening of chemical libraries. In the current era, the majority of the assays used either target-based analysis or molecule validation including cell-based approaches. A wide variety of assays were being designed for highly sensitive detection techniques and miniaturized protocols were also included. Cell-based assays were also used for addressing G protein coupled receptors (GPCRs), kinases, nuclear hormone receptors, ion channels and many more useful studies are possible [124]. A widely used approach for cell-based assays employs the use of immortalized cells for HTS. Immortalized cells are engineered recombinant cells that are capable of expressing a specific molecular target, whose functionality can be assayed for detecting and quantifying using automated detection systems. HTS assays with a cellular approach is a potentially reliable system for detecting specific molecules. For example, in a specific case of GPCRs measurement of functional responses of receptor-compound interaction, complex cell responses, protein translocations, kinase pathway activation can be done with HTS technology [125, 126]. Immortalized cells were generally used to screen library of chemicals, small molecules, drugs and so on, and also for multiple screening of drug molecules for identification, standardization and validation, which helps in establishing a compound's potency, specificity and reliability. Immortalized cells were also used to study the pharmacokinetics of drugs [127]. Immortalized cells are transfected with molecular targets, which aids in an unlimited number of cell divisions to grow for unlimited quantities; thus providing a robust platform for HTS studies. In addition to cloning, expression systems were used for investigating expression of drug based targets, engineering protein expressions, reporter proteins and so on [126, 128]. However, some of the major limitations of immortalized cells, such as an immortalized cellular system, is not a reliable platform with regard to reflecting the human *in vivo* physiological system. The major reason for this is due to genetic modifications, which vary the morphology, molecular and phenotypic characteristics of immortalized cells in comparison to human native cells [129]. This investigation was confirmed in GPCR studies; due to the use of transfected immortalized cells, expression levels of GPCRs were frequently elevated much higher than physiologically healthy cells. Overexpression of GPCRs has changed the ratio of GPCRs to G protein, consequently affecting the efficacy of receptor activation. G protein activation also affects the pharmacological feature of GPCR. Overall, cellular microenvironments effects of G protein properties influences the signalling pathways in HTS assays [127]. Thus an artificial micro-environment from immortalized cells increases the abnormal expression of receptors, cellular effects, cellular signalling systems and these drug screening data obtained from immortalized cellular systems clearly show several issues that hinder the reliability of drug candidates to be considered for human testing and subsequent therapeutic applications.

Due to the complications of recombinant immortalized cells, primary mammalian cells have gained interest in HTS assays. Primary cells show better relevance with human physiological systems in understanding scenario of diseases, cell-cell interactions and signalling. Additionally, a library of novel drugs could be characterized and screened using primary cell systems that can predict functional activity and provide more

reliable results than immortalized cells [130]. Primary cells consist of cells derived from embryonic tissues including neuronal cultures and cells from adult tissues such as hepatocytes. Primary cells are used to evaluate the activity of chemicals, screening pharmaceutical drug discovery studies in recombinant studies to study the expression of desired gene using vector systems and implementation in transgenic animals. In comparison with immortalized cells, primary cells are a reliable platform to perform functional studies such as intracellular calcium levels, migration of proteins, cell-cell interactions, signalling pathways as well as electrophysiological changes that measure membrane functionality [131, 132]. Procurement, propagation and long-term functionality are the major challenges faced in primary mammalian cell culture system. Due to limited donors, procurement of a sufficient number primary cells is a challenging task. Primary cells are very sensitive and fragile in handling when compared to immortalized cells; they cannot be propagated for an unlimited number of passages. When primary cells are cultured for higher passages, there are chances of losing the functional properties. Thus, compensated qualities of primary cells restrict them to be employed in highly sensitive assays and scaling up of technology for mass production for HTS. Collective assay data points from the primary cell-based assays might show false positive results due to the changes in molecular or functional features. Primary cells are not suitable for HTS applications for several reasons. Primary cells cultured from adult tissues result in terminal differentiation or senescence, ultimately the functional stability. For optimal and reliable results use of embryonic tissue originated primary cells are recommended for HTS and drug discovery studies.

3.5 Role of Stem Cells in High Throughput Screening for Toxicity Prediction

The introduction of new screening technologies has led to the advancement of stem cell biology and their application in screening toxic chemical drugs in large scale via HTS. Prior to investing in the formulation of compounds and commercialization for human use, screening of drug chemicals for risk-free applications in drug discovery and drug development is very important. Cell-based high-throughput screening (HTS) technologies are being implemented for validation of molecular as well as cellular targets. Stem cells clearly provide unlimited source of reproducible cells from a very wide range of tissues, additionally they are accurate in simulating human *in vivo* physiological conditions. Ultimately, providing a realistic platform with more clinically relevant phenotypes than those currently available for predicting and screening toxicity [133]. Stem cells from adult tissue/embryonic origin/induced pluripotent stem cells (iPSCs)-derived cells have shown reliable results in comparison with any other cell source. More specifically, embryonic stem cells have shown remarkable applications in drug development and toxicity screening [134–136]. A study highlighted the ability of human embryonic stem cells derived fibroblasts for assessing cytotoxic and genotoxic levels of drugs when compared with immortalized cells [137–139]. Drugs and chemicals primarily enter the body after exposing to skin's epithelial barrier. Reactivity of drugs with skin cells is one of vital steps in development of risk-free drugs. Studies have been reported hESC derived keratinocytes being implemented for validating the toxic part of biomaterials and chemicals. Furthermore, hESC-derived progenies such as pericytes, neurons, cardiomyocytes and hepatocytes have proved to be a potential platform for evaluation of toxicity of drugs [140]. Thus, use of stem cells in drug discovery is now exponentially progressing from a nascent level of development to a point where the cells may begin to be widely employed in HTS, lead optimization and profiling. HTS technology can be used in industry as well as in academic research to scale up the process of screening and testing of drugs.

3.5.1 Applications of Stem Cells in Cardiotoxicity HTS

An exponential increase in adverse effects of drugs and pharmaceutical products has resulted in an increase in the cost pressure on consumers. Valid reasons for failure in optimal drug synthesis is lack of appropriate platform to test/validate the drug efficacy, thus leading to poor preclinical assessment [141].

Cardiotoxicity is an important assay to consider for screening the adverse side effects and detrimental effects of new drugs on cardiac tissue. A realistic and reliable platform that can reflect the human milieu would be beneficial for screening drugs with animal-free assays. The *in vitro* production of human cardiomyocytes from stem cells will be a potential approach for drug screening and discovery [142]. Among stem cell, pluripotent stem cells show up self-renewal features for unlimited divisions, thus can be valuable cell source for scaling up assays by HTS [143]. Unfortunately, existing differentiation protocols of cardiomyocytes from human pluripotent stem cells needs optimization and characterization in terms of protein expression as well as in functional aspects. The *in vitro* rhythmic cardiac contractions are measured by using patch clamp assay or multi-electrode arrays, thus representing promising results in predicting cardiotoxicity [144]. However, some aspects of existing differentiation protocols still remain challenging. Variations in purity of cardiomyocytes derived from pluripotent cells is one of the major limitations [145]. Regardless of limitations, stem cells have gained high interest and demand in the field of regenerative medicine, drug discovery, development, toxicity screening for long-term goal and mass production.

3.5.2 Applications of Stem Cells in Hepatotoxicity HTS

In pharmaceutical and drug industries, adverse effects of drug on liver functioning are pressing issues for a patient's safety and drug's commercial value. Several detrimental side effects of drugs and chemicals have reduced the market value as well as represented as a major reason for liver toxicity. Hepatotoxic drugs cause liver injury and in most cases hepatic failure after long-term exposure [146]. Development in predictive toxicology to understand safety risk and mechanisms still remains a challenging task. Various factors, such as lack of an appropriate cell source in models, are restricting improvisation in establishing novel model for screening hepatotoxicity [147, 148]. HepaRG cells derived from liver carcinoma are the only source of cells that are able to differentiate into hepatocyte-like cells and biliary cells. Functionally and morphologically HepaRG cells are comparable to normal primary hepatocytes. Surprisingly, 85% of the genes expressed in primary human hepatocytes are comparable with differentiated HepaRG cells and also show stability of several weeks under confluency. Thus proves, HepaRG also as an efficient platform for drug metabolism, toxicity studies and drug discovery. [149]. In addition to primary hepatocytes and transformed cell lines, stem cells either from embryo/reprogrammed or from adult tissues have proved to be promising source for supplying an unlimited number of hepatocytes. Implementing hepatocyte-like cells derived from stem cells for assessing toxicity implies the metabolically competency and expression of xenobiotic metabolic enzymes at consistent levels in comparison with liver or in primary hepatocyte cultures. Functional analysis has been proven by only a few studies, whereas most studies are restricted to a few cytochromes P450 [150]. Among stem cell sources, embryonic stem cell-derived hepatocyte-like cells have represented the highest activity [151, 152]. The degree of *in vitro* differentiation of embryonic stem cells or the occurrence of genetic and epigenetic abnormalities during serial passages was improvised by FACS differentiated hepatocytes-like cells for specific expressed markers and studies were performed to investigate the functional stability and possible transdifferentiation [20, 153]. Regulation of cytokines, growth factors, micro-supplements in the form of cocktails or sequential treatments have also been considered during differentiation process. However, obtaining matured functional hepatocytes from stem cells remains a challenging task. For a decade, active stem cell research has demonstrated differentiation potential of adult-stem cells, embryonic stem cell and induce pluripotent stem cells in obtaining functional hepatocytes under *in vitro* milieu [154]. Scaling up of differentiation technology is also a major challenge, which could be overcome by high expertise in handling cells and regulated growth conditions in mass production. Application of stem cell derived-hepatocyte like cells in HTS can be in high demand for screening suspected drugs for hepatotoxicity.

3.5.3 Applications of Stem Cells in Neurotoxicity HTS

The nervous system consists of two sub-systems; central nervous system (CNS) and peripheral nervous system (PNS). The CNS is composed of the brain and spinal cord, PNS composed of ganglia and peripheral nerves. Neurons and neuroglial cells are two main types of cells found in nervous system. Neurons are very sensitive and specialized cells found in the body. Neurons are responsible for reception, integration, transmission and storage of information [155]. Neurobiology field has provided an *in vitro* cell culture platform to understand mechanism and pathways involved in functional aspects of neurons. Investigation of toxic effects of chemical, biological and physical agents on the nervous system and/or behaviour during development and maturation is termed *neurotoxicology*. For evaluating potential neurotoxic substances, various *in vitro* models have been established including primary cells, cell lines and cloned/immortalized cells [156]. However, existing cell sources highlight the lack of appropriateness and reliability, thus existing cell sources are not efficient enough to simulate human *in vivo* physiological system and lead to unacceptable outcomes.

Discovery of stem cells and implementing in the field of toxicology for developing *in vitro* platforms has been improvised and solved. It is important to establish an efficient platform for screening toxicity and efficacy of drug candidates. Among stem cells, pluripotent stem cells either from embryo or chemically induced cells have shown the unlimited supply of neurons without compromising the functionality of mature neurons. Human stem cell-derived neurons can be a potential source for investigating neurotoxic compounds and also provides a powerful tool for understanding the neurobiology pathways, mechanisms and cellular events [157, 158]. In addition to drug screening and toxicity testing, stem cell derived-neurons represent a pre-clinical model for reducing/replacing animal experiments and making a cost effective pre-clinical model [159–161]. However, a major challenging aspect of stem cell approach is mass production of stem cell derived neurons with consistent functional stability. Particularly in the case of reprogrammed cells, protocols need to be standardized and optimized to regulate the yield of reprogrammed cells with minimal inter-batch variability. Scaling up the technology and bringing towards HTS for drug discovery, disease models such as Parkinson's disease and also neurotoxic assessment of chemicals will be beneficial steps in the field of toxicology [162, 163]. Strategies of HTS, HTS importance in toxicology and improvements in cellular and non-cellular studies with involvement of stem cell sources can increase the impact and reliability of results, ultimately raising commercial value and attrition rate of drugs developed. The HTS model of neurotoxicity will be a valuable tool in pharmaceutical and biomedical industries.

3.6 Conclusion

Social and ethical concerns have made scientists (toxicologists and chemists) achieve new bioassay profiling data with relevance to toxicology studies. Upcoming novel methods show a significant impact in accessing toxicity profile of new chemicals. Nowadays, scientists are looking for high-throughput pre-clinical safety assessment for new biochemical and biomedical compounds [164]. High-throughput assays show reliability and are more promising than conventional methods. HTS assays were analysed by the means of high content screening and bio-imaging techniques. Functional HTS are able to measure a compound's functionality and specificity with a target molecule/protein, such as flow of ions through potassium ion channels. On the other hand, non-functional HTS assays are more focused on measuring the accuracy in binding a target protein, measuring fluorescence activity and other analytical methods. However, major drawbacks of existing HTS assays are cell source, lack of human relevance and loss in cell functionality, thus making the screening platform unreliable. The stem cell approach for HTS toxicity has the potential to overcome the existing backlogs as well as improve standards to gain good commercial value for drugs to benefit industries and companies. Scientists are also aiming to miniaturize specific organ/tissue systems of human body in a chip by introducing

the concept of lab-on-a-chip for HTS [165, 166]. Additionally, HTS toxicology research augmented with stem cell research will establish a new horizon to the field of medicine to understand human pathophysiological conditions as well as diagnose diseases. The supreme goal of augmenting stem cells with HTS toxicity will be guided by humane innovation towards validation, regulatory acceptance and implementation of non-animal test methods to construct a patient-concerned preclinical platform on a large scale within a short time span.

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Author's Contribution

HHK prepared the manuscript. GS contributed in critical reviews and in preparation of manuscript, TC supported with suggestions for improvising final manuscript draft. All authors read and approved the final manuscript.

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4

Human Pluripotent Stem Cells for Toxicological Screening

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4.1 Introduction

The development of xenobiotics, driven by the demand for therapeutic, domestic, and industrial uses continues to grow. However, along with this increasing demand is the risk of xenobiotic-induced toxicity. During their whole life, human beings are exposed to xenobiotics, such as diverse chemical substances, pharmaceutical drugs, and other potentially hazardous chemical and physical environmental factors. The toxicology tests of these xenobiotics are concerned with human health. At present, many *in vitro* and *in vivo* models are used to analyze the potential toxic effects of pharmaceutical drugs and chemicals (Stummann et al. 2009; Vojnits and Bremer 2010).

For quite a long period of time, whole animal models *in vivo* (*in vivo* tests) have been used as the main prediction methods to judge sample damages on human health. However, these *in vivo* tests are problematic, because toxicity tests using animals are not representative for human beings due to species-specific pharmaco-toxicological effects. Sometimes, the benefits demonstrated in the animal models do not come out to be beneficial in humans, for example, the SOD (superoxidase dimutase) gene associated with ALS (Amyotrophic Lateral Sclerosis) allowed the identification of Vitamin E and Creatine to be relievers of the diseased phenotype which failed to cause any improvements in humans (Shefner et al. 2004). Another aspect is the high number of animals that are required for toxicology testing (Gilbert 2010). In addition, animal tests are not adequately standardized. So these *in vivo* tests are time-consuming, laborious, expensive, and, specifically, require the use of high numbers of laboratory animals.

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Therefore, alternative *in vitro* screening methods have been developed to detect potential hazardous effects of chemicals or drugs on embryonic development. *In vitro* cellular models that accurately reflect human physiology have the potential to improve the prediction of drug toxicity early in the development pipeline (Fabre et al. 2014) and would provide a cost-effective approach for testing other sources of xenobiotics exposure, including food additives, cosmetics, pesticides, and industrial chemicals (Crofton et al. 2011; Judson et al. 2014). While human cellular test systems would overcome the problem of species specificity, *in vitro*-cultured human immortalized cell lines do not represent normal cell types, and human primary cells cultured *in vitro* usually lose their tissue-specific functions. And the short life cycle of primary human cell culture *in vitro* limits their application in tissue formation, regeneration, and toxicology tests.

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), provide invaluable resources for regenerative medicine. The distinct advantages that these cells have over adult stem cells are their unlimited proliferative capacity and much more extensive differentiation potential. As such, cardiac, hepatic, and neural differentiation of hPSCs could provide a promising cell source for regenerative medicine and cell therapy applications. The recent applications of pluripotent stem cells and their derivatives in toxicology and drug research provide new alternatives to the standard routine tests performed by the industry and offer new strategies for chemical safety assessment (Laustriat et al. 2010; Trosko and Chang 2010).

This review will give a brief overview of the unique properties of different types of pluripotent stem cells for toxicological studies with special consideration of hPSCs and their use in developmental as well as in cardiac, hepatic, and neural toxicology and introduce the current development of biomarkers or methods in embryotoxic and developmental toxicity tests using hPSC.

4.2 The Biological Characteristics of hPSCs

4.2.1 The Biological Characteristics of hESCs

hESCs were isolated from pre-implantation embryos and cultured in an undifferentiated status (Thomson et al. 1998). hESCs possess the capacity to differentiate *in vitro* into cells that encompass all three embryonic germlines (Jones and Thomson 2000). Therefore, their biological characteristics of ES cell determines its broad application prospects. While initial hopes raised by hESCs were directed towards their potential use in replacement therapies, the focus of research has now shifted to the development of pluripotent cell-based models for drug research, toxicological test systems and disease modeling *in vitro*. hESCs are believed to provide a more closely associated analogue for toxicity assessments pertaining to early human embryonic development (West et al., 2010). However, utilization of hESCs is ethically controversial because it involves the destruction of human embryos. The use of hESCs in research is laden with ethical issues regarding personhood, justice toward human kind and human dignity that are associated with the use of human life in its earliest form, the embryo.

4.2.2 The Biological Characteristics of hiPSCs

Recently developed induced pluripotent stem cells (iPSCs) are increasingly attracting wide interest in toxicology test. The iPS cells are generated from the induction of expression of transcription factors associated with pluripotency, allowing a differentiated somatic cell to reverse its condition to the embryonic stage. Currently, it is generally accepted in the scientific community that hiPSCs are highly similar if not virtually identical to hESCs in terms of their morphology, surface marker expression, feeder dependence, and *in vivo* teratoma formation capacity (Yu et al. 2007).

hiPSCs offer various advantages as compared with hESCs (Kazuki et al. 2010). Firstly, the iPS technology avoids the embryo destruction or manipulation to generate pluripotent cells, therefore, are exempt from ethical implication surrounding embryonic stem cell use. Secondly, iPSCs are generated from the somatic cells of one's own body and hence there is no risk of immunorejection of these autologous cells (Guha et al. 2013). Thirdly, it is feasible to throughput screen for predicting toxicity/therapeutic responses of newly developed drugs. The concept of using iPSCs to predict toxicology and therapeutic responses of drugs in based on the property of iPSCs to continuously self-renew, which make it possible to generate libraries, and their ability to give rise to all types of body cells make them suitable to be used for prediction of toxicity and possible side effects of newly developed drugs in different body cells (Wobus and Loser 2011). Lastly, a personalized approach for administration of drugs can be developed using iPSCs. As hiPSCs are derived from individual patients, these offer scientists an opportunity for modeling diseases on a patient-by-patient basis. This enables screening the genomic differences between individuals that may help in the progression of disease, and the screening of pharmacological agents to find the ideal one for each individual (Chun et al. 2011).

4.3 Screening of Embryotoxic Effects using hPSCs

4.3.1 Screening of Embryotoxic Effects using hESCs

In the past, primarily mouse embryonic stem cells (mESC) were used for the stem cell test which provided ground work for development of the Embryonic Stem Cell Test (EST) (Scholz et al. 1999). The EST was designated more recently as one of the most promising validated *in vitro* tests for developmental toxicity prediction (Adler et al. 2008a). The development of hESC-based *in vitro* systems for testing embryotoxicity of chemical compounds would be a significant progress. The use of hESCs would enhance the predictivity of *in vitro* assays and avoid problems associated with the interpretation of results from animal-based assays in a human context. For example, species-specific differences between mouse and human preimplantation development, such as in DNA methylation, and DNA repair and expression of genes involved in drug metabolism may hamper the correct interpretation of animal studies for human beings (Krtolica et al. 2009). The use of hESC-based test systems could avoid incorrect classification of chemicals due to inter-species variations and, consequently, would increase the safety of consumers and patients.

Several proof-of-concept studies show that hESCs would be a suitable model for analyzing developmental toxicity (Adler et al. 2008a, 2008b; Zdravkovic et al. 2008; Krishnamoorthy et al. 2010). In some of these studies, potential toxic effects of agents and noxes (such as certain chemical compounds, radiation, ethanol, or cigarette smoke) on the viability and integrity of hESCs were investigated (Cao et al. 2008; Mehta et al. 2008; Bueno et al. 2009; Flora and Mehta 2009). This kind of study may allow conclusions on the effects of certain hazards on the preimplantation embryo *in vivo*.

The novel methods and biomarkers to screen toxic chemicals during the developmental process using undifferentiated human embryonic stem cells were developed in recent years (Table 4.1).

Jung and co-workers assessed the developmental toxicity of embryotoxic chemicals, 5-fluorouracil, indomethacin, and non-embryotoxic penicillin G using an Affymetrix GeneChips (Jung et al. 2015). After treatment with 5-fluorouracil, indomethacin, and penicillin G, they observed a remarkable convergence in the degree of upregulation of development, cell cycle, and apoptosis-related genes by gene expression profiles using an Affymetrix GeneChips. Taken together, these results suggest that embryotoxic chemicals have cytotoxic effects, and modulate the expression of ES cell markers as well as development-, cell cycle-, and apoptosis-related genes that have pivotal roles in undifferentiated hES cells.

In another study, a metabolic biomarker-based *in vitro* assay utilizing human embryonic stem cells was developed to identify the concentration of test compounds that perturbs cellular metabolism in a manner

Table 4.1 *Summary of current development of biomarkers or methods in embryotoxic test using hPSCs*

Xenobiotics	Cell lines	Biomarkers or method	Key references
5-fluorouracil indomethacin	hES	pluripotent ES cell markers gene expression profiles using an Affymetrix GeneChips	(Jung et al. 2015)
46 compounds	WA09 hES line	metabolite biomarkers (ornithine and cystine)	(Palmer et al. 2013)
Mitomycin C	chHES-3, chHES-8, chHES-22 and chHES-254	chromosomal aberrations; copy number variation using Affymetrix SNP 6.0 arrays	(Zhou et al. 2015)
BDE-209	FY-hES-10 and FY-hES-26	pluripotent genes expression; DNA methylation and microRNA expression	(Du et al. 2015)
71 drug-like compounds	H9 and LSJ-1	nuclear translocation of the transcription factor SOX17	(Kameoka et al. 2014)
Sodium valproate Ascorbic acid Thalidomide	hiPSCs	the inhibition of cardiac differentiation the cytotoxicity to hiPSCs the cytotoxicity to human dermal fibroblasts	(Aikawa et al. 2014)
Cr(VI); hydrogen peroxide; doxorubicin	hiPSCs	DNA damage responses	(Lu et al. 2013)

indicative of teratogenicity (Palmer et al. 2013). Metabolomic data from hES cells culture media were used to assess potential biomarkers for development of a rapid *in vitro* teratogenicity assay. Two metabolite biomarkers (ornithine and cystine) were identified as indicators of developmental toxicity. The predictivity of the new assay was evaluated using a separate set of test compounds. The new assay identified the potential developmental toxicants in the test set with 77% accuracy (57% sensitivity, 100% specificity). The assay had a high concordance (≥75%) with existing *in vivo* models, demonstrating that the new assay can predict the developmental toxicity potential of new compounds as part of discovery phase testing and provide a signal as to the likely outcome of required *in vivo* tests.

In addition to the gene expression and metabolism index, genetic stability and epigenetic indicators also were used in toxicity evaluation with hPSC. In a recent study, the copy number variation changes of the hES cells were investigated. Mitomycin C (MMC), a DNA damage agent, is widely used for preparation of feeder cells in many laboratories (Zhou et al. 2015). In this study, it was found that the copy number variation changes of the hES cells maintained on MMC-inactivated feeders (MMC-feeder) were significantly more than those cultured on gamma-inactivated feeder (IR-feeder) cells using Affymetrix SNP 6.0 arrays. Furthermore, DNA damage response (DDR) genes were down-regulated during long-term culture in the MMC-containing system, leading to DDR defect and shortened telomeres of hES cells, a sign of genomic instability. Therefore, MMC-feeder and MMC-induced genomic variation present an important safety problem that could limit such hES from being applied for future clinic use and drug screening.

Environment factors have varying effects on DNA methylation depending on the nature of the exposure, such as during prenatal life or adulthood, with environmental toxicants capable of modifying DNA methylation (Terry et al. 2011). DNA methylation regulates pluripotent gene expression during embryonic development and differentiation in hESCs (Yeo et al. 2007). In another study, Du and colleagues found decabromodiphenyl ether (BDE-209) exposure could decrease pluripotent genes expression via epigenetic

regulation in hESCs (Du et al. 2015). They found an increased methylation of the OCT4 promoter and increased miR-145/miR-335 levels ponded to decreased OCT4 levels in BDE-209-treated hESCs. These findings suggest that BDE-209 inhibits OCT4 expression via epigenetic regulation in hESCs.

While the detection of individual compounds toxicity, hESCs also can be used for high throughput screening of drugs toxicity. Kameoka et al. examined 71 drug-like compounds with known *in vivo* effects, including thalidomide using Human ES cell lines H9 and LSJ-1 (Kameoka et al. 2014). A threshold of 5 muM demonstrated 94% accuracy (97% sensitivity and 92% specificity). Furthermore, 15 environmental toxicants with physicochemical properties distinct from small molecule pharmaceutical agents were examined and a similarly strong concordance with teratogenicity outcomes from *in vivo* studies was observed. Finally, to assess the suitability of the hEST for high-throughput screens, a small library of 300 kinase inhibitors was tested, demonstrating the hEST platform's utility for interrogating teratogenic mechanisms and drug safety prediction. Thus, the hEST assay is a robust predictor of teratogenicity and appears to be an improvement over existing *in vitro* models. Together, these studies suggest that hES cells may be useful for testing the toxic effects of chemicals that could impact the embryonic developmental stage.

4.3.2 Screening of Embryotoxic Effects using hiPSCs

iPSCs also possess unique properties of self-renewal and differentiation to many types of cell lineage. Hence, they could replace the use of embryonic stem cells and may overcome the various ethical issues regarding the use of embryos in research and clinics. Several publications have reviewed studies where iPSCs have been used to evaluate for toxicity and effects of many toxic compounds (different chemical compounds, pharmaceutical drugs, other hazardous chemicals, or environmental conditions), which are encountered by humans and newly designed drugs (Heng et al. 2009; Sison-Young et al. 2012; Scott et al. 2013; Singh et al. 2015).

In a recent study, the predictive abilities of hiPSCs and mouse embryonic stem cells in toxicity test were compared (Aikawa et al. 2014). Three endpoints: the inhibition of cardiac differentiation, the cytotoxicity to hiPSCs, and the cytotoxicity to human dermal fibroblasts, according to the mEST were assessed. It was found that thalidomide was classified as a Class 2 agent, with weak embryotoxicity, by the mEST criteria, and was classified as Category 3 embryotoxic based on hiPSC criteria. Ascorbic acid was classified as a Class 1/ Category 1, non-embryotoxic agent, based on both criteria. This test system is thus considered to have a much greater predictive ability than the mEST. In this present study, the true extent of developmental toxicity of thalidomide in humans was detected by using *in vitro* test system based on the hiPSCs for the first time. This humanized test system may be able to more accurately predict the developmental toxicity of drug candidates at an early stage in drug development by combining the mEST criteria with the hiPSCs criteria.

Lu et al. characterized the effect of Cr(VI), a well-known genotoxic agent and environmental carcinogen, on major molecular components of DNA damage response pathways in human iPS cells (Lu et al. 2013). They compared the effect of Cr(VI) on human iPS cells with two established cell lines, Tera-1 (teratoma origin) and BEAS-2B (lung epithelial origin). They also studied the effect of hydrogen peroxide and doxorubicin on modulating DNA damage responses in these cell types. They demonstrated that ATM and p53 phosphorylation is differentially regulated in human iPS cells compared with Tera-1 and BEAS-2B cells after exposure to various genotoxic agents. Their data reveal some unique features of DNA damage responses in human iPS cells.

In vitro organogenesis is now becoming a realistic goal of stem cell biology, as one can obtain an unlimited number of pluripotent stem cells through reprogramming technology. One practical challenge is to develop a four-dimensional (4-D) stem cell culture system whereby multiple progenitors communicate in a spatiotemporal manner, as observed during *in vivo* organogenesis. Takebe and Taniguchi summarize the potential for emerging culture platforms in the future application of induced pluripotent stem cell (iPSC)-derived miniature organs by recapitulating early embryogenesis (Takebe and Taniguchi 2014).

4.4 The Potential of hPSC-Derived Neural Lineages in Neurotoxicology

A large number of potential neurotoxic compounds pose serious health hazards to the human nervous system, yet little is known about how exposure to compounds will impact human neural function and development. The developing human brain is particularly susceptible to environmental toxicants, and the damage induced by neurotoxins can range from onset of neurodevelopment disorders to long-lasting neurological impairments (Tofighi et al. 2011). With growing awareness and concern regarding the potential neurotoxicity of environmental contaminants, prescription drugs, and industrial chemicals, much attention and effort has been directed towards neurotoxicology.

Establishment of efficient stepwise differentiation protocols for directing hPSCs into specific cell lineages is an essential prerequisite for both therapeutic and basic research applications. There are many methods to initiate neural differentiation of hPSCs including conventional techniques such as embryoid body (EB) formation (from dissociated suspension culture) and cocultivation with stromal cell lines (Denham and Dottori 2011). More recently, studies on small molecule inducers of neuronal differentiation have focused on directing hPSCs into each of the four major specific neuronal sublineages, that is, dopaminergic, serotonergic, GABAergic, and cholinergic/motor neurons (summarized in Yap et al. (2015).

Hence, there is much interest in developing small-molecule based differentiation protocols for deriving specific neuronal sublineages from hPSCs, for *in vitro* modeling of neurodegenerative diseases, as well as for pharmacological screening of new drugs to treat these diseases.

4.4.1 The Challenge of hPSCs-Derived Neural Lineages in Neurotoxicology Applications

In order to demonstrate that human iPSC and their differentiated derivatives is critical for properly understanding the human nervous system biology including neurotoxicity and development neurotoxicology. Schulpen et al. compared mouse and human neural ESTn assays for neurodevelopmental toxicity as to regulation of gene expression during cell differentiation in both assays (Schulpen et al. 2015). They found that both systems nicely illustrated successful neural differentiation of ESC *in vitro*, with both commonalities and unique gene expression changes occurring in response to time and VPA exposure. The mESTn assay clearly shows a more specific neurodevelopmental differentiation pattern, whereas the hESTn also showed differentiation of cell types originating from other germ layers as well. Both these assays have their advantages, for instance as to specificity and species of origin. With further optimization of the human assay, for example removal of the need for a feeder layer for undifferentiated hES cells and opening the possibility for subculture from controlled cell suspensions, the assay may become technically easier.

The another challenge is to demonstrate the reliability of these *in vitro* methods by correlating the *in vitro* produced results to the available *in vivo* data. Pei et al. report on the comparative cytotoxicity of 80 compounds (neurotoxicants, developmental neurotoxicants, and environmental compounds) in iPSC as well as isogenic iPSC-derived neural stem cells (NSC), neurons, and astrocytes. All compounds were tested over a 24-h period at 10 and 100 μM, in duplicate, with cytotoxicity measured using the MTT assay. Of the 80 compounds tested, 50 induced significant cytotoxicity in at least one cell type; per cell type, 32, 38, 46, and 41 induced significant cytotoxicity in iPSC, NSC, neurons, and astrocytes, respectively. Four compounds (valinomycin, 3,3',5,5'-tetrabromobisphenol, deltamethrin, and triphenyl phosphate) were cytotoxic in all four cell types. Retesting these compounds at 1, 10, and 100 μM using the same exposure protocol yielded consistent results as compared with the primary screen. Using rotenone, we extended the testing to seven additional iPSC lines of both genders; no substantial difference in the extent of cytotoxicity was detected among the cell lines. Finally, the cytotoxicity assay was simplified by measuring luciferase activity using lineage-specific luciferase reporter iPSC lines, which were generated from the parental iPSC line. This article is part of a journal Special Issue entitled SI: PSC and the brain (Pei et al. 2015).

Table 4.2 *Summary of current development of biomarkers or methods in neurotoxicology using hPSCs-derived neural lineages*

Xenobiotics	Cell lines	Biomarkers or method	Key references
valproic acid carbamazepine	hESC (WA09-DL11) mESC (ES-D3)	Gene expression by Affymetrix microarray	(Schulpen et al. 2015)
VPA	hESCs from Kyoto university	expressions of the taget genes by quantitative real-time PCR	(Ehashi et al. 2014)
valproic acid	hESCs lines (HUES1 and H9)	microarray transcriptomic data	(Colleoni et al. 2014)
Lead	WA09 hESC line	DNA methylation by HumanMethylation450 BeadChip	(Senut et al. 2014)
Propofol	H1 hESCs line	microRNAs, including miR-21	(Twaroski et al. 2014)
Valproate trichostatin A	H9 hESCs line	Histone acetylation Histone H3 lysine methylation	(Balmer et al. 2014)
ketamine	iPSC line	mitochondrial membrane potential ATP	(Ito et al. 2015)
36 compounds	hN2™ hESCs line	High Content Analysis	(Wilson et al. 2014)
6 Chemicals	iPSC	Multiparametric Live-Cell Toxicity Assay	(Sirenko et al. 2015)
12 chemicals	hNP1 cells from WA09 hESC	multiplexed assay suitable for high-throughput screening	(Druwe et al. 2015)
Nanoparticles	WA09 hESC	a 3-D neurosphere system	(Hoelting et al. 2013)
60 chemicals	H1 hESCs line	h-derived neural tissue constructs with vascular networks and microglia	(Schwartz et al. 2015)

4.4.2 The New Biomarkers in Neurotoxicology using hPSC-Derived Neural Lineages

There are many indicators to evaluate neurodevelopmental toxicity using the pluripotent stem cell neural differentiation model. The new biomarkers used in neurotoxicitology in recent two years are reviewed here (Table 4.2).

4.4.2.1 Gene Expression Regulation

Schulpen and co-workers studied gene expression regulation after valproic acid and carbamazepine exposure in a human embryonic stem cell-based neurodevelopmental toxicity assay (Schulpen et al. 2015). During neural differentiation the cells were exposed, for either 1 or 7 days, to noncytotoxic concentration ranges of valproic acid (VPA) or carbamazepine (CBZ), antiepileptic drugs known to cause neurodevelopmental toxicity. The effects observed on gene expression and correlated processes and pathways were in line with processes associated with neural development and pharmaceutical mode of action. They found that VPA showed a higher number of genes and molecular pathways affected than CBZ. The response kinetics differed between both compounds, with CBZ showing higher response magnitudes at day 1, versus VPA at day 7. In another study, they found that valproic acid (VPA) and carbamazepine (CBZ) exposure during hESTn differentiation led to concentration-dependent reduced expression of betaIII-tubulin, Neurogin1 and Reelin. In parallel VPA caused an increased gene expression of Map2 and Mapt, which is possibly related to the neural protective effect of VPA (Schulpen et al. 2015). They demonstrated the potential and biological relevance of the application of this hESC-based differentiation assay in combination with transcriptomics.

Ehashi et al. also investigated expressions of the taget genes by quantitative real-time PCR and compared during differentiation of human ESCs into neurons with or without VPA (Ehashi et al. 2014). They observed

that neural development-related genes such as DCX, ARX, MAP2, and NNAT showed a more than two-fold expression in VPA exposure group. They suggested that the neural development-related genes may help to elucidate the teratogenic effects of VPA and might be a useful tool to analyze embryotoxic potential of chemicals in humans.

Recently, transcriptomic approaches associated to toxicogenomic database analysis have given the possibility to screen, annotate, and cluster high numbers of genes and to identify the molecular changes that univocally mark the toxicity induced processes or are indicative of the early initiating events that lead to cellular toxicity. Colleoni et al. compared microarray transcriptomic data derived from two different hESCs lines (HUES1 and H9) exposed to valproic acid while applying the same differentiation protocol (Colleoni et al. 2014). The results showed that molecular changes in the processes of neural development, neural crest migration, apoptosis, and regulation of transcription, indicated a good correspondence with the available *in vivo* data. They also described common toxicological signatures and provided an interpretation of the observed qualitative differences referring to known biological features of the two hESC lines.

4.4.2.2 Epigenetic Markers

Several recent studies investigated developmental neurotoxicity using epigenetic techniques. Senut et al. investigated the effects of physiologically relevant concentrations of Pb (from 0.4 to 1.9 μM) on the capacity of human embryonic stem cells (hESCs) to progress to a neuronal fate (Senut et al. 2014). They found that neither acute nor chronic exposure to Pb prevented hESCs from generating neural progenitor cells (NPCs). NPCs derived from hESCs chronically exposed to 1.9 μM Pb throughout the neural differentiation process generated 2.5 times more TUJ1-positive neurons than those derived from control hESCs. Pb exposure of hESCs during the stage of neural rosette formation resulted in a significant decrease in the expression levels of the neural marker genes PAX6 and MSI1. DNA methylation studies of control, acutely treated hESCs and NPCs derived from chronically exposed hESCs using the Illumina HumanMethylation450 BeadChip demonstrated that Pb exposure induced changes in the methylation status of genes involved in neurogenetic signaling pathways. Their data shows that exposure to Pb subtly alters the neuronal differentiation of exposed hESCs and that these changes could be partly mediated by modifications in the DNA methylation status of genes crucial to brain development.

MicroRNAs (miRs) are endogenous, non-coding RNA molecules that act to regulate nearly every cellular process through inhibition of target messenger RNA expression. MicroRNAs have been implicated to play important roles in many different disease processes, including neurological diseases (Hebert et al. 2008; Johnson et al. 2008). However, the role of microRNAs in anesthetic-induced neurotoxicity has yet to be studied. Twaroski et al. found propofol dose and exposure time dependently induced significant cell death in the human embryonic stem cell-derived neurons and downregulated several microRNAs, including miR-21 (Twaroski et al. 2014). Overexpression of miR-21 and knockdown of Sprouty 2 attenuated the increase in terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end labeling-positive cells following propofol exposure. In addition, miR-21 knockdown increased the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end labeling-positive cells by 30%. This is the first time that a role for microRNAs in the mechanism of anesthetic-induced neurotoxicity has been established. These data suggest that human embryonic stem cell-derived neurons represent a promising *in vitro* human model for studying anesthetic-induced neurotoxicity and the propofol-induced cell death may occur via a signal transducer and activator of transcription 3/miR-21/Sprouty 2-dependent mechanism.

Alteration of histone deacetylase (HDAC) activity has been associated with several long-term health consequences, ranging from Alzheimer's disease (Graff et al. 2012). Balmer detected the role of histone acetylation and methylation as epigenetic switch between reversible and irreversible drug effects in the neural developmental toxicity of valproate and trichostatin A (Balmer et al. 2014). They found that Histone acetylation

(primary MoA) increased quickly and returned to baseline after 48 h. Histone H3 lysine methylation at the promoter of the neurodevelopmental regulators PAX6 or OTX2 was increasingly altered over time. Methylation changes remained persistent and correlated with neurodevelopmental defects and with effects on PAX6 gene expression, also when the drug was washed out after 3–4 days. They suggested that drug exposures altering only acetylation would lead to reversible transcriptome changes (indicating MoA), and challenges that altered methylation would lead to irreversible developmental disturbances.

4.4.2.3 Mitochondrial Function

Ito et al. reported an *in vitro* model for assessing the neurotoxicity of ketamine in iPSC-derived neurons with mitochondrial membrane potential and ATP as indicators (Ito et al. 2015). Twenty-four-hour exposure of iPSC-derived neurons to 500 μM ketamine resulted in a 40% increase in caspase 3/7 activity ($P<0.01$), 14% increase in ROS production ($P<0.01$), and 81% reduction in mitochondrial membrane potential ($P<0.01$), compared with untreated cells. Lower concentration of ketamine (100 μM) decreased the ATP level (22%, $P<0.01$) and increased the NADH/NAD+ ratio (46%, $P<0.05$) without caspase activation. Transmission electron microscopy showed enhanced mitochondrial fission and autophagocytosis at the 100 μM ketamine concentration, which suggests that mitochondrial dysfunction preceded ROS generation and caspase activation. Their data indicated that the initial mitochondrial dysfunction and autophagy may be related to its inhibitory effect on the mitochondrial electron transport system, which underlies ketamine-induced neural toxicity.

4.4.3 The New Methods in Neurotoxicology using hPSC-Derived Neural Lineages

4.4.3.1 High-Throughput Methods

High-throughput methods are useful for rapidly screening large numbers of chemicals for biological activity, including the perturbation of pathways that may lead to adverse cellular effects. High Content Analysis (HCA) technology has emerged as a technology well-suited for high-throughput assessment of neuron-specific endpoints (Culbreth et al. 2012). Wilson et al. developed a multiplexed HCA-based neurotoxicity screening assay using a combination of four detection reagents per well – antibodies against βIII-Tubulin and the phosphorylated form of the neurofilament subunit NF-H (pNF-H), the DNA-binding Hoechst 33342 nuclear dye, and Mitotracker® Red CMXRos (Wilson et al. 2014). This assay was run using a test set of 36 chemicals in differentiated SH-SY5Y, PC-12, and hN2™ cells, and results were compared with those obtained under the same conditions with the MTT and LDH assays. Data showed that multiparametric High Content Analysis of differentiated neuronal cells is feasible and represents a highly effective method for obtaining large quantities of robust data on the neurotoxic effects of compounds compared with cytotoxicity assays like MTT and LDH. Significant differences were observed between the responses to compounds across the three cellular models tested, illustrating the heterogeneity in responses to neurotoxicants across different cell types. This study provides data strongly supporting the use of cellular imaging as a tool for neurotoxicity assessment in differentiated neuronal cells, and provides novel insights into the neurotoxic effects of a test set of compounds upon differentiated neuronal cell lines and human embryonic stem cell-derived neurons.

Another group reported high-content imaging and analysis methods to assess multiple phenotypes in human iPSC-derived neuronal cells (Sirenko et al. 2015). Specifically, they optimized cell culture, staining, and imaging protocols in a 384-well assay format and improved laboratory workflow by designing a one-step procedure to reduce assay time and minimize cell disturbance. Phenotypic readouts include quantitative characterization of neurite outgrowth and branching, cell number and viability, as well as measures of adverse effects on mitochondrial integrity and membrane potential. They reported concentration-response effects of

selected test compounds on human iPSC-derived neuronal cells and illustrate how the proposed methods may be used for high-content high-throughput compound toxicity screening and safety evaluation of drugs and environmental chemicals.

Druwe and colleagues examined the utility of a high-throughput assay to detect chemical-induced apoptosis in mouse or human neuroprogenitor cells, as well as differentiated human neurons derived from induced pluripotent stem cells (Druwe et al. 2015). Apoptosis was assessed using an assay that measures enzymatic activity of caspase-3/7 in a rapid and cost efficient manner. The results show that all three commercially available models generated a robust source of proliferating neuroprogenitor cells, and that the assay was sensitive and reproducible when used in a multi-well plate format. There were differences in the response of rodent and human neuroprogenitor cells to a set of chemicals previously shown to induce apoptosis *in vitro*. Neuroprogenitor cells were more sensitive to chemical-induced apoptosis than differentiated neurons, suggesting that neuroprogenitor cells are one of the cell models that should be considered for use in a developmental neurotoxicity screening battery.

4.4.3.2 Three-Dimensional (3-D) Culture

The 3D model was characterized for neural marker expression revealing robust differentiation toward neuronal precursor cells, and gene expression profiling suggested a predominantly forebrain-like development. Hoelting and coworkers developed a human embryonic stem cell (hESC)-derived three-dimensional (3D) *in vitro* model that allows for testing of potential developmental neurotoxicants (Hoelting et al. 2013). Altered neural gene expression due to exposure to non-cytotoxic concentrations of the known developmental neurotoxicant, methylmercury, indicated that the 3D model could detect developmental neurotoxicity. To test for specific toxicity of nanoparticles (NPs), chemically inert polyethylene NPs (PE-NPs) were chosen. They penetrated deep into the 3D structures and impacted gene expression at non-cytotoxic concentrations. NOTCH pathway genes such as HES5 and NOTCH1 were reduced in expression, as well as downstream neuronal precursor genes such as NEUROD1 and ASCL1. FOXG1, a patterning marker, was also reduced. They showed that their 3D neurosphere model detected the known developmental neurotoxicity compound, methylmercury, with good sensitivity and that expression of neurodevelopmental genes was affected upon NP exposure.

Differentiating pluripotent stem cells *in vitro* have proven useful for the study of developmental toxicity. Schwartz et al. reported human pluripotent stem cell-derived neural constructs to predict neural toxicity (Schwartz et al. 2015). Human embryonic stem (ES) cell-derived neural progenitor cells, endothelial cells, mesenchymal stem cells, and microglia/macrophage precursors were combined on chemically defined polyethylene glycol hydrogels and cultured in serum-free medium to model cellular interactions within the developing brain. The precursors self-assembled into 3D neural constructs with diverse neuronal and glial populations, interconnected vascular networks, and ramified microglia. Replicate constructs were reproducible by RNA sequencing (RNA-Seq) and expressed neurogenesis, vasculature development, and microglia genes. Linear support vector machines were used to construct a predictive model from RNA-Seq data for 240 neural constructs treated with 34 toxic and 26 nontoxic chemicals. This predictive model was evaluated using two standard hold-out testing methods: a nearly unbiased leave-one-out cross-validation for the 60 training compounds and an unbiased blinded trial using a single hold-out set of 10 additional chemicals. The linear support vector produced an estimate for future data of 0.91 in the cross-validation experiment and correctly classified 9 out of 10 chemicals in the blinded trial. Pairing such actively perfused devices with the vascularized 3D neural constructs described here may promote more advanced differentiation and growth, whereas the incorporation of blood–brain barrier function would be beneficial for investigating the delivery of therapeutic agents and could improve the predictive accuracy of our model by delivering toxic chemicals in a more physiologically relevant manner.

4.5 The Potential of hPSC-Derived Cardiomyocytes in Cardiotoxicity

Human induced pluripotent stem cells (hiPSCs) have emerged as a novel tool for drug discovery and therapy in cardiovascular medicine. Cardiac lineage- and tissue-specific human pluripotent stem cell (hPSC) reporter lines have been valuable for the identification, selection, and expansion of cardiac progenitor cells and their derivatives, and for our current understanding of the underlying molecular mechanisms. The potential usefulness of iPSC-derived cardiomyocytes (iPSC-CMs) in drug development as well as in drug toxicity testing is discussed in several reviews (Mercola et al. 2013; Mordwinkin et al. 2013; Sharma et al. 2013; Sinnecker et al. 2014).

4.5.1 The Challenge of hPSC-Derived Cardiomyocytes in Cardiotoxicology Applications

Scott evaluated both human induced pluripotent stem cell-derived CMs (hiPSC-CMs) and rat neonatal CMs (rat CMs) on the xCELLigence Cardio system which uses impedance technology to quantify CM beating properties in a 96-well format (Scott et al. 2014). Forty-nine compounds were tested in concentration-response mode to determine potency for modulation of CM beating, a surrogate biomarker for contractility. In comparison with *in vivo* contractility effects, hiPSC-CM impedance had assay sensitivity, specificity, and accuracy values of 90, 74, and 82, respectively. These values compared favorably to values reported for the dog CM optical assay (83, 84, and 82%) and were slightly better than impedance using rat CMs (77, 74, and 74%). The potency values from the hiPSC-CM and rat CM assays spanned four orders of magnitude and correlated with values from the dog CM optical assay ($r(2) = 0.76$ and 0.70, respectively). The Cardio system assay has >5× higher throughput than the optical assay. Thus, hiPSC-CM impedance testing can help detect the human cardiotoxic potential of novel therapeutics early in drug discovery, and if a hazard is identified, has sufficient throughput to support the design-make-test-analyze cycle to mitigate this liability.

4.5.2 The New Biomarkers in Cardiotoxicology using hPSC-Derived Cardiomyocytes

The currently available techniques for the safety evaluation of candidate drugs are usually cost intensive and time consuming and are often insufficient to predict human relevant cardiotoxicity (Table 4.3).

Table 4.3 *Summary of current development of biomarkers or methods in cardiotoxicity test using hPSCs-derived CMs*

Xenobiotics	Cell lines	Biomarkers or method	Key references
doxorubicin	Cellartis® Pure hES-CM	cardiac specific troponin T (cTnT) gene expression of GDF15	(Holmgren et al. 2015)
24 drugs	hiPSC-CM	Structural and functional screening	(Doherty et al. 2015)
Ponatinib	hiPSC-CM	Structural and functional screening	(Talbert et al. 2015)
Doxorubicin Daunorubicin mitoxantrone	hiPSC-CM	Global gene expression using microarrays and bioinformatics tools	(Chaudhari et al. 2015)
12 drugs	hiPSC-CM	Multiple parameters using multi-electrode array	(Qu and Vargas 2015)
TAB, aflatoxin B1, miodarone, menadione, sunitinib, crizotinib	hiPSC-CM	multiparametric screening format including calcium flux, high-content cell imaging of mitochondrial integrity, and reactive oxygen species (ROS)	(Grimm et al. 2015)

4.5.2.1 Gene Expression

Holmgren and coworkers investigated the toxic effects of doxorubicin exposure in cardiomyocytes derived from human embryonic stem cells (hESC). They identified novel biomarkers for doxorubicin-induced toxicity in human cardiomyocytes derived from hESCs (Holmgren et al. 2015). A dose-dependent pattern was observed for the release of cardiac specific troponin T (cTnT) after 1 day and 2 days of treatment with doxorubicin. Except global transcriptional profiles in the cells, the cTnT release was used as a measurement of acute cardiotoxicity due to doxorubicin. However, for the late onset of doxorubicin-induced cardiomyopathy, cTnT release might not be the most optimal biomarker. On the other hand, the gene expression of *GDF15* is a more sensitive marker compared to cTnT measurement and, as such, might be a more predictive biomarker than the conventional biomarkers used for anthracycline-mediated cardiovascular events.

Another group reported a multi-parameter *in vitro* screen in human stem cell-derived cardiomyocytes to identify ponatinib or multiple drug class-induced structural and functional cardiac toxicity (Doherty et al. 2015; Talbert et al. 2015). Multi-parameter including structural cardiac (as shown by actin cytoskeleton damage, mitochondrial stress, cell death, and troponin secretion), and cardiac cell beating were used as cardiac toxicity biomarkers. Their studies show that a multi-parameter approach examining both cardiac cell health and function in hiPS-CM provides a comprehensive and robust assessment that can aid in the determination of potential cardiac liability.

Chaudhari et al. developed an *in vitro* repeated exposure toxicity methodology allowing the identification of predictive genomics biomarkers of functional relevance for drug-induced cardiotoxicity in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) (Chaudhari et al. 2015). Global gene expression changes were studied using microarrays and bioinformatics tools. Analysis of the transcriptomic data revealed early expression signatures of genes involved in formation of sarcomeric structures, regulation of ion homeostasis, and induction of apoptosis. Eighty-four significantly deregulated genes related to cardiac functions, stress, and apoptosis were validated using real-time PCR. The expression of the 84 genes was further studied by real-time PCR in hiPSC-CMs incubated with daunorubicin and mitoxantrone, further anthracycline family members that are also known to induce cardiotoxicity. A panel of 35 genes was deregulated by all three anthracycline family members and can therefore be expected to predict the cardiotoxicity of compounds acting by similar mechanisms as doxorubicin, daunorubicin, or mitoxantrone. The identified gene panel can be applied in the safety assessment of novel drug candidates as well as available therapeutics to identify compounds that may cause cardiotoxicity.

4.5.2.2 Multi-Electrode Array

Evaluation of stem cell-derived cardiomyocytes (SC-CM) using multi-electrode array (MEA) has attracted attention as a novel model to detect drug-induced arrhythmia. Qu et al. evaluated proarrhythmia risk in human induced pluripotent stem cell-derived cardiomyocytes using the Maestro MEA Platform (Qu and Vargas 2015). Multiple parameters, including field potential duration (FPD), Na(+) slope, Na(+) amplitude, beat rate (BR), and early after-depolarization (EAD) were recorded. Minimum effective concentrations (MEC) that elicited a significant change were calculated. They found that FPD and EAD were unable to distinguish torsadogenic from benign compounds, Na(+) slope and amplitude could not differentiate Na(+) channel blockade from hERG blockade, BR had an inconsistent response to pharmacological treatment, and that hiPSC-CM were, in general, insensitive to IKs inhibition. A ratio was calculated that relates MEC for evoking FPD prolongation, or triggering EAD, to the human therapeutic unbound Cmax (MEC/Cmax). The key finding was that the ratio was sensitive, but specificity was low. Consistently, the ratio had high positive predictive value and low negative predictive value. Their datas showed that MEA recordings of hiPSC-CM were sensitive for FPD and EAD detection, but unable to distinguish agents with low- and high-risk for TdPs. Although some published reports

suggested great potential for MEA recordings in hSC-CM to assess preclinical cardiac toxicity, their data implies that this model would have a high false-positive rate in regard to proarrhythmic risk.

4.5.3 High-Throughput Methods

Cell-based high-content screening (HCS) assays have become an increasingly attractive alternative to traditional *in vitro* and *in vivo* testing in pharmaceutical drug development and toxicological safety assessment. The time- and cost-effectiveness of HCS assays, combined with the organotypic nature of human induced pluripotent stem cell (iPSC)-derived cells, open new opportunities to employ physiologically relevant *in vitro* model systems to improve screening for potential chemical hazards.

Grimm and coworkers used two human iPSC types, cardiomyocytes and hepatocytes, to test various high-content and molecular assay combinations for their applicability in a multiparametric screening format (Grimm et al. 2015). Effects on cardiomyocyte beat frequency were characterized by calcium flux measurements for up to 90 min. Subsequent correlation with intracellular cAMP levels was used to determine if the effects on cardiac physiology were G-protein-coupled receptor dependent. In addition, they utilized high-content cell imaging to simultaneously determine cell viability, mitochondrial integrity, and reactive oxygen species (ROS) formation in both cell types. Kinetic analysis indicated that ROS formation is best detectable 30 min following initial treatment, whereas cytotoxic effects were most stable after 24 h. For hepatocytes, high-content imaging was also used to evaluate cytotoxicity and cytoskeletal integrity, as well as mitochondrial integrity and the potential for lipid accumulation. Lipid accumulation, a marker for hepatic steatosis, was most reliably detected 48 h following treatment with test compounds. Overall, their results demonstrate how a compendium of assays can be utilized for quantitative screening of chemical effects in iPSC cardiomyocytes and hepatocytes, and enable rapid and cost-efficient multidimensional biological profiling of toxicity.

These results are promising and suggest that hiPSC-CMs may be a faithful model for predicting drug serving as a test bed for discovering new drug treatments for cardiotoxicity.

4.6 The Potential of hPSC-Derived Hepatocytes in Hepatotoxicity

Primary human hepatocytes (PHHs) are a limited resource for drug screening, their quality for *in vitro* use can vary considerably across different lots, and a lack of available donor diversity restricts our understanding of how human genetics affect drug-induced liver injury. Induced pluripotent stem cell-derived human hepatocyte-like cells (iPSC-HHs) could provide a complementary tool to PHHs for high-throughput drug screening, and ultimately enable personalized medicine. Potential applications of induced pluripotent stem cells (iPSCs) in hepatology research were reviewed in several publications (Subba Rao et al. 2013; Sun et al. 2015).

4.6.1 The Challenge of hPSCs-Derived Hepatocytes in Hepatotoxicology Application

Human-induced pluripotent stem cell-derived hepatocytes (hiPSC-Hep) hold great potential as an unlimited cell source for toxicity testing in drug discovery research. However, little is known about mechanisms of compound toxicity in hiPSC-Hep. Sjogren compared the toxic responses of Human-induced pluripotent stem cell-derived hepatocytes (hiPSC-Hep) primary cryopreserved human hepatocytes (cryo-hHep) and the hepatic cell lines HepaRG and Huh7 when treated with staurosporine and acetaminophen (Sjogren et al. 2014). They found that for studying compounds initiating apoptosis directly hiPSC-Hep may be a good alternative to cryo-hHep. Furthermore, for compounds with more complex mechanisms of toxicity involving metabolic activation, such as acetaminophen, our data suggest that the cause of cell death depends on a balance between factors controlling death signals and the drug-metabolizing capacity.

Ulvestad and coworkers compared the expression and function of important drug metabolizing cytochrome P450 (CYP) enzymes and transporter proteins in human embryonic and induced pluripotent stem cell-derived hepatocytes (hESC-Hep and hiPSC-Hep) with cryopreserved human primary hepatocytes (hphep) and HepG2 cells (Ulvestad et al. 2013). They found that the stable expression and function of CYPs and transporters in hESC-Hep and hiPSC-Hep for at least 1 week opens up the possibility to reproducibly perform long term and extensive studies; for example chronic toxicity testing, in a stem cell-derived hepatic system.

Drug-induced liver injury (DILI) remains a great challenge and a major concern during late-stage drug development. Induced pluripotent stem cells (iPSC) represent an exciting alternative *in vitro* model system to explore the role of genetic diversity in DILI, especially when derived from patients who have experienced drug-induced hepatotoxicity. The development and validation of the iPSC-derived hepatocytes as an *in vitro* cell-based model of DILI is an essential first step in creating more predictive tools for understanding patient-specific hepatotoxic responses to drug treatment. Lu performed extensive morphological and functional analyses on iPSC-derived hepatocytes from a commercial source (Lu et al. 2015). They found that iPSC-derived hepatocytes exhibit many of the key morphological and functional features of primary hepatocytes, including membrane polarity and production of glycogen, lipids, and key hepatic proteins, such as albumin, asialoglycoprotein receptor and alpha1-antitrypsin. They maintain functional activity for many drug-metabolizing enzyme pathways and possess active efflux capacity of marker substrates into bile canalicular compartments. Whole genome-wide array analysis of multiple batches of iPSC-derived cells showed that their transcriptional profiles are more similar to those from neonatal and adult hepatocytes than those from fetal liver. Results from experiments using prototype DILI compounds, such as acetaminophen and trovafloxacin, indicate that these cells are able to reproduce key characteristic metabolic and adaptive responses attributed to the drug-induced hepatotoxic effects *in vivo*. Overall, this novel system represents a promising new tool for understanding the underlying mechanisms of idiosyncratic DILI and for screening new compounds for DILI-related liabilities.

4.6.2 The New Biomarkers in Hepatotoxicology using hPSC-Derived Hepatocytes

Emerging hepatic models for the study of drug-induced toxicity include pluripotent stem cell-derived hepatocyte-like cells and complex hepatocyte-non-parenchymal cellular coculture to mimic the complex multicellular interactions that recapitulate the niche environment in the human liver. However, a specific marker of hepatocyte perturbation, required to discriminate hepatocyte damage from non-specific cellular toxicity contributed by non-hepatocyte cell types or immature differentiated cells is currently lacking, as the cytotoxicity assays routinely used in *in vitro* toxicology research depend on intracellular molecules which are ubiquitously present in all eukaryotic cell types (Table 4.4).

Kia and coworkers demonstrate that microRNA-122 (miR-122) detection in cell culture media can be used as a hepatocyte-enriched *in vitro* marker of drug-induced toxicity in homogeneous cultures of hepatic cells, and a cell-specific marker of toxicity of hepatic cells in heterogeneous cultures such as HLCs generated from various differentiation protocols and pluripotent stem cell lines, where conventional cytotoxicity assays using generic cellular markers may not be appropriate (Kia et al. 2015). They found that the sensitivity of the miR-122 cytotoxicity assay is similar to conventional assays that measure lactate dehydrogenase activity and intracellular adenosine triphosphate when applied in hepatic models with high levels of intracellular miR-122, and can be multiplexed with other assays. MiR-122 as a biomarker also has the potential to bridge results in *in vitro* experiments to *in vivo* animal models and human samples using the same assay, and to link findings from clinical studies in determining the relevance of *in vitro* models being developed for the study of drug-induced liver injury.

Alpers-Huttenlocher syndrome (AHS), a neurometabolic disorder caused by mutations in mitochondrial DNA polymerase gamma (POLG), is associated with an increased risk of developing fatal VPA hepatotoxicity.

Table 4.4 *Summary of current development of biomarkers or methods in hepatotoxicity test using hPSCs-derived hepatocytes*

Xenobiotics	Cell lines	Biomarkers or method	Key references
Acetaminophen diclofenac	HUES7 hESC line Shef-3 hESC line	MiR-122	(Kia et al. 2015)
VPA	AHS iPSCs-Hep	mitochondrial-dependent apoptotic pathway	(Li et al. 2015)
47 drug	iPSC-HH	multiple functional endpoints micropatterned co-cultures (iMPCCs)	(Ware et al. 2015)
omeprazole rifampicin	ES cell-derived hepatocytes	metabolic function in aggregate culture	(Sengupta et al. 2014)
Amiodarone aflatoxin B1 troglitazone Ximelagatran	hiPS-HEP	long-term exposure to toxic drugs	(Holmgren et al. 2014)

However, the mechanistic link of this clinical mystery remains unknown. Li et al. used the hepatocyte-like derived from AHS iPSCs (AHS iPSCs-Hep) to evaluate the hepotoxicity of VPA (Li et al. 2015). AHS iPSCs-Heps were more sensitive to VPA-induced mitochondrial-dependent apoptosis than controls, showing more activated caspase-9 and cytochrome c release. Strikingly, levels of both soluble and oligomeric optic atrophy 1, which together keep cristae junctions tight, were reduced in AHS iPSCs-Hep. Furthermore, POLG mutation cells showed reduced POLG expression, mitochondrial DNA (mtDNA) amount, mitochondrial adenosine triphosphate production, as well as abnormal mitochondrial ultrastructure after differentiation to hepatocyte-like cells. Superoxide flashes, spontaneous bursts of superoxide generation, caused by opening of the mitochondrial permeability transition pore (mPTP), occurred more frequently in AHS iPSCs-Hep. Moreover, the mPTP inhibitor, cyclosporine A, rescued VPA-induced apoptotic sensitivity in AHS iPSCs-Hep. This result suggested that targeting mPTP opening could be an effective method to prevent hepatotoxicity by VPA in AHS patients. AHS iPSCs-Hep are more sensitive to the VPA-induced mitochondrial-dependent apoptotic pathway, and this effect is mediated by mPTP opening.

4.6.3 The New Methods in Hepatotoxicology using hPSC-Derived Hepatocytes

4.6.3.1 iPSC-HH-Based Micropatterned Co-Cultures (iMPCCs) with Murine Embryonic Fibroblasts

Primary human hepatocytes (PHHs) are a limited resource for drug screening, their quality for *in vitro* use can vary considerably across different lots, and a lack of available donor diversity restricts our understanding of how human genetics affect drug-induced liver injury (DILI). Induced pluripotent stem cell-derived human hepatocyte-like cells (iPSC-HHs) could provide a complementary tool to PHHs for high-throughput drug screening, and ultimately enable personalized medicine. Ware reported that previously developed iPSC-HH-based micropatterned cocultures (iMPCCs) with murine embryonic fibroblasts could be amenable to long-term drug toxicity assessment (Ware et al. 2015). iMPCCs, created in industry-standard 96-well plates, were treated for 6 days with a set of 47 drugs, and multiple functional endpoints (albumin, urea, ATP) were evaluated in dosed cultures against vehicle-only controls to enable binary toxicity decisions. They found that iMPCCs correctly classified 24 of 37 hepatotoxic drugs (65% sensitivity), while all 10 non-toxic drugs tested were classified as such in iMPCCs (100% specificity). On the other hand, conventional confluent cultures of iPSC-HHs failed to detect several liver toxins that were picked up in iMPCCs. Results for DILI detection in iMPCCs were remarkably similar to published data in PHH-MPCCs (65% vs 70% sensitivity) that were

dosed with the same drugs. Furthermore, iMPCCs detected the relative hepatotoxicity of structural drug analogs and recapitulated known mechanisms of acetaminophen toxicity *in vitro*. In conclusion, their data showed that iMPCCs could provide a robust tool to screen for DILI potential of large compound libraries in early stages of drug development using an abundant supply of commercially available iPSC-HHs.

4.6.3.2 Suspension Culture of Aggregates of ES Cell-Derived Hepatocytes

Early phase drug development relies on primary human hepatocytes for studies of drug metabolism, cytotoxicity, and drug-drug interactions. However, primary human hepatocytes rapidly lose metabolic functions *ex vivo* and are refractory to expansion in culture and thus are limited in quantity. Hepatocytes derived from human pluripotent stem cells (either embryonic stem (ES) or induced pluripotent stem (iPS) cells), have the potential to overcome many of the limitations of primary human hepatocytes, but to date the use of human pluripotent stem cell-derived hepatocytes has been limited by poor enzyme inducibility and immature metabolic function. Sengupta and coworkers presented a simple suspension culture of aggregates of ES cell-derived hepatocytes that compared to conventional monolayer adherent culture significantly increases induction of CYP 1A2 by omeprazole and 3A4 by rifampicin (Sengupta et al. 2014). Using liquid chromatography-tandem mass spectrometry, they further showed that ES cell-derived hepatocytes in aggregate culture convert omeprazole and rifampicin to their human-specific metabolites. They also showed that these cells convert acetaminophen (APAP) to its cytotoxic metabolite (N-acetyl-p-benzoquinone imine (NAPQI)), although they fail to perform APAP glucuronidation. In summary, they showed that human pluripotent stem cell-derived hepatocytes in aggregate culture display improved enzymatic inducibility and metabolic function and is a promising step toward a simple, scalable system, but nonetheless will require further improvements to completely replace primary human hepatocytes in drug development.

4.6.3.3 Long-Term Exposure to Toxic Drugs

Late-stage attrition in the pharmaceutical industry is to a large extent caused by selection of drug candidates using nonpredictive preclinical models that are not clinically relevant. The current hepatic *in vivo* and *in vitro* models show clear limitations, especially for studies of chronic hepatotoxicity. Holmgren evaluated the potential of using hPSC-derived hepatocytes for long-term exposure to toxic drugs (Holmgren et al. 2014). The differentiated hepatocytes were incubated with hepatotoxic compounds for up to 14 days, using a repeated-dose approach. The hPSC-derived hepatocytes became more sensitive to the toxic compounds after extended exposures and, in addition to conventional cytotoxicity, evidence of phospholipidosis and steatosis was also observed in the cells. This was the first report of a long-term toxicity study using hPSC-derived hepatocytes, and the observations support further development and validation of hPSC-based toxicity models for evaluating novel drugs, chemicals, and cosmetics.

4.7 Future Challenges and Perspectives for Embryotoxicity and Developmental Toxicity Studies using hPSCs

As discussed previously, hiPSCs and hESCs have advanced as cell sources when applied in embryotoxicity and developmental toxicology studies. In particular, some progress has been made in the determination of endpoints suitable for determination of embryotoxicity in differentiating hPSCs, and proof-of-concept was provided that hPSC-based *in vitro* systems may be useful to predict human-specific developmental toxicity.

However, there are numerous problems to be solved before hPSC-based assays can be implemented into routine procedures for developmental toxicity testing of drugs and chemicals. Firstly, the generation of iPSCS

itself has many issues associated it like incorporation of vectors into host genomes, and the dependence on the various factors like usage of small molecules, single or multiple vectors, source of cells, which make the generation of iPSCs a risky task and hence, more about iPSCs needs to be explored to make it easier to generate them and to be able to apply them to various other important and attractive areas of medical sciences for their proper utilization to achieve the advantages that use of iPSCs can have. In particularly, the reprogramming efficiency remains low until now. Secondly, the predictivity, sensitivity, and specificity of the respective test systems have still to be shown for a wider panel of drugs and chemicals. So far, only few substances have been tested in the different approaches. Thirdly, the low efficiency of specific differentiative cells generation was one of the issues arising when it comes to toxicity test. The establishment of reliable and reproducible differentiation procedures that can be performed in a high-throughput format is necessary. Fourthly, the differentiated cells from hPSCs are not a homogenous group of cells. For example, even fully differentiated hiPSC-CMs display heterogeneity in terms of being at various stages of development and maturity. Single cell transcriptional profiling confirms heterogeneity between individual hiPSCs (Narsinh et al. 2011). Twenty-eight pluripotency related transcripts and 14 differentiated state related transcripts were measured in hiPSCs and hESCs. In aggregate, they had the same mean levels of transcript expression, but the frequency distribution in hiPSCs was much broader, suggesting greater inter-cell line variability (Narsinh et al. 2011). This heterogeneity impacts on the generation of reproducible results from these cells. In addition, the limitation of hPSCs derived cells is that they retain certain stem-cell and fetal characteristics. For example, commercially available hiPSC-CMs (iCells), after being thawed and cultured for 3–5 days, were compared with human fetal and human adult cardiomyocytes (Guo et al. 2011). The expression levels of genes for sarcomeric proteins in hiPSC-CMs more closely resembled those in fetal cardiomyocytes. Specifically, expression of MYL2, MYH7, TNNI3, SCN5A, and KCNH2 more closely resembled fetal levels; whereas expression of MYH6 resembled adult levels. The expression of MYL7, MYH7 and TNNI3 was less than that seen in both fetal and adult cardiomyocytes (Guo et al. 2011). Last but not least, there are differences in the epigenetic regulation in hPS-derived cells and somatic cells. hESC-Hep have a limited drug metabolism ability, which restricts their use for *in vitro* hepatotoxicity testing. This is because the majority of CYP genes involved in drug metabolism, including CYP1A2, CYP2C9, CYP2C19, CYP3A4, CYP2D6, and CYP2E1, are lowly expressed in hECS-Hep. Park et al. investigated their epigenetic regulation in terms of DNA methylation and histone modifications in hESC-Hep and hPH (Park et al. 2015). They found that DNA methylation and histone modifications of regulatory regions of CYP genes differed between hPH and hESC-Hep. These differences were associated with inhibitory regulation of CYP genes in hESC-Hep. Inhibition of DNMTs and HDACs increased the transcription of CYP genes in hESC-Hep, but these increased transcripts were not comparable with that in hPH. Further studies are required to improve the expression and activity of CYP enzymes by epigenetic regulations. These findings showed that expression of CYP genes is modulated by controlling epigenetic modification enzymes, such as DNMTs and HDACs.

Here, we have summarized recent developments regarding the application of hiPSCs and hESCs in embryotoxicity and developmental toxicity research. Although several problems exist before hPSC-based assays can be implemented into routine procedures for developmental toxicity testing of drugs and chemicals, hPSCs are likely the most appropriate *in vitro* model for embryonic development toxicity screening.

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5

Effects of Culture Conditions on Maturation of Stem Cell-Derived Cardiomyocytes

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5.1 Introduction

Toxicology and safety concerns are the primary reasons that clinical entities either fail to complete the drug development phase or are withdrawn from the market after approval. Cardiovascular toxicity has been reported to occur more often than other types of drug-induced toxicities (Laverty et al., 2011) with life-threatening cardiac arrhythmias being the most commonly reported form. The Food and Drug Administration (FDA) currently recommends that all new drug candidates be evaluated for their ability to induce QT prolongation and Torsades de Pointes, a rare but possibly fatal ventricular arrhythmia. The International Conference on Harmonization (ICH) S7B Guidance, "The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals" was approved in 2005 (www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S7B/Step4/S7B_Guideline.pdf). This guidance includes electrocardiogram (ECG) in an *in vivo* animal model as well as *in vitro* screening for effects on the voltage gated eag related subfamily H, member 2 potassium channel (*KCNH2*), more commonly known as *HERG*. The guidance has been very successful at eliminating drugs that might induce Torsades de Pointes before they are marketed; however, a number of limitations have been noted (Darpo et al., 2014). QT prolongation may be induced by methods other than blocking the KCNH2 encoded potassium channel, and some compounds may block the channel at therapeutic concentrations but do not cause arrhythmias. Additionally, due to the sensitivity of the assay, many promising drug candidates may be abandoned early in the development process.

Recognizing these problems, a think tank that was sponsored by the FDA, Cardiac Safety Research Consortium, and Health and Environmental Sciences Institute met in 2013 to assess the current safety testing

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protocol. The outcome of the meeting was the proposal for a Comprehensive *in vitro* Proarrhythmia Assay (CiPA) testing paradigm. This paradigm has three components; the first is the evaluation of drug effects on multiple cardiac ionic currents in heterologous expression systems. *In silico* models are then used to determine the potential for drug-induced repolarization instability, and lastly the models are tested in an *in vitro* system such as cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs; Sager et al., 2014).

The differentiation of cardiomyocytes from hiPSCs is a fairly new technology, and a thorough understanding of the technology, as well as its advantages and limitations are necessary prior to its use in regulatory applications. Currently, there are a few commercial sources for hiPSC-derived cardiomyocytes, and there may be variability in the responses of the cells to various drugs. Additionally, the cells that are available commercially at present include only limited types of disease models which could be useful in examining efficacy of new entities in treating cardiovascular diseases. To date, studies have used a variety of protocols to induce differentiation of hiPSCs or human embryonic stem cells (hESCs) to cardiomyocytes. These methods often yield a mixture of cardiomyocytes and other cells types. Some of the protocols result in cardiomyocytes that appear more atrial-like while other protocols result in cardiomyocytes that are more ventricle-like or even nodal-like. Additionally, optimization of the differentiation protocols for individual cell lines is required in order to produce the highest yield of cardiomyocytes.

However, a more significant feature of the cardiomyocytes differentiated via these various protocols is that their phenotype is more similar to that of an immature, or fetal, cardiomyocyte than to the phenotype of an adult cardiomyocyte. For examination of many cardiac diseases as well as for application of cardiomyocytes for regenerative medicine, it is necessary that the cardiomyocytes have a more mature phenotype. Ebert et al. (2015) have suggested that defining culture conditions that produce more mature cardiomyocytes is a "most immediate need." However, it is not yet known if more mature cardiomyocytes are needed for safety evaluation of drug candidates.

Characteristics of fetal and mature cardiomyocytes have been compared (van den Heuvel et al., 2014; Yang et al., 2014; Karakikes et al., 2015). Briefly, fetal cardiomyocytes are small, rounded cells that do not display organized sarcomeres. Gap junction protein, alpha 1, 43 kDa (GJA1; Table 5.1), more commonly known as connexin 43, is distributed around the periphery of the cell rather than near the ends of the cells as in the gap junctions connecting adjacent mature cardiomyocytes. Fetal cells are generally more proliferative and have only a single nucleus. These cells also contain few mitochondria that do not have very well developed cristae on their inner membranes. The fetal cells generally rely on glycolysis for energy production and express different ion channels than those that are present in mature cardiomyocytes. This leads to spontaneous contractions and a decreased upstroke velocity and a higher resting membrane potential in action potential measurements (van den Heuvel et al., 2014; Yang et al., 2014; Karakikes et al., 2015). Mature cardiomyocytes, in contrast, are rod shaped elongated cells with organized and aligned sarcomeres. Their proliferative capacity is diminished, and the cells are multinucleated. Gap junctions are located primarily at the short ends of the cells where they connect to neighboring cells. Mitochondria increase in number, and metabolism switches primarily to beta oxidation of fatty acids. Electrophysiology and calcium handling are also different due to differential expression of ion channels and Ca^{2+} handling proteins (Yang et al., 2014; Karakikes et al, 2015).

The transcriptomes of human fetal hearts have been compared to those of cardiomyocytes differentiated from hiPSCs (van den Berg et al., 2015; Synnergren et al., 2012). Both studies found that the gene expression profiles of the hiPSC-derived cardiomyocytes were similar to those of hearts from human fetuses. van den Berg et al. (2015) also observed that the gene expression profile of hiPSC-derived cardiomyocytes that had been induced to undergo a more enhanced maturation in culture resembled hearts from second trimester fetuses; less mature cardiomyocytes appeared to be more similar to hearts from first trimester fetuses.

Recent reviews have discussed the methods that have been used in attempts to produce a more mature hiPSC-derived cardiomyocyte (Yang et al., 2014; Veerman et al., 2015). These methods include lengthening the culture time, providing electrical stimulation, or mechanical stress, altering the culture substrate either by changing the stiffness of the substrate or providing physical cues by patterns in or on the substrate, co-culturing

Table 5.1 *Current human gene names and their associated synonyms**

Gene Name	Synonyms
ACTA2 Actin, Alpha 2, Smooth Muscle, Aorta	Alpha-Cardiac Actin, Alpha-Actin 2, *ACTSA*, *MYMY5*, *AAT6, ACTVS*
ACTC1 Actin, Alpha, Cardiac Muscle 1	*ACTC*, Alpha-Cardiac Actin, *CMD1R*, *LVNC4*, *CMH11*
ACTN1 Actinin, Alpha 1	Alpha-Actinin-1, Actinin 1 Smooth Muscle, Non-Muscle Alpha-Actinin-1, *BDPLT15*
ATP2A2 ATPase, Ca++ Transporting, Cardiac Muscle, Slow Twitch 2	*SERCA2*, *ATP2B*, *DAR*, Cardiac Ca2+ ATPase
CACNA1C Calcium Channel, Voltage-Dependent, L Type, Alpha 1C Subunit	*CCHL1A1*, *CACNL1A1*, *CACH2*
CASQ2 Calsequestrin 2 (Cardiac Muscle)	Calsequestrin 2 Fast-Twitch Cardiac Muscle, *PDIB2*
GATA4 GATA Binding Protein 4	*TACHD*, *ASD2*, *VSD1*, Transcription Factor GATA-4
GJA1 Gap Junction Protein, Alpha 1, 43 kDa	Connexin 43, *CX43*, *GJAL*, *ODDD*, *AVSD3*, *HLHS1*
ITGA7 Integrin, Alpha 7	
ITGB1 Integrin, Beta 1 (Fibronectin Receptor, Beta Polypeptide, Antigen CD29 includes *MDF2*, *MSK12*)	*FNRB*, *MSK12*, *MDF2*, CD29 Antigen, VLA-4 Subunit Beta, Fibronectin Receptor Subunit Beta, *GPIIA*
KCNH2 Potassium Channel, Voltage Gated Eag Related Subfamily H, Member 2	*HERG*, Ether-A-Go-Go-Related Gene Potassium Channel, Eag-Related Protein 1, *ERG1*, *LQT2*
KCNJ12 Potassium Channel, Inwardly Rectifying Subfamily J, Member 12	*KCNJN1*, Inward Rectifier K(+) Channel Kir2.2v, *IRK2*, ATP-Sensitive Inward Rectifier Potassium Channel 12, Kir2.2v, *HIRK1*
MYH6 Myosin, Heavy Chain 6, Cardiac Muscle, Alpha	Alpha-Myosin Heavy Chain, Myosin Heavy Chain 6, MYHCA, MYHC, Alpha-MHC
MYH7 Myosin, Heavy Chain 7, Cardiac Muscle, Beta	Beta-Myosin Heavy Chain, *MYHCB*, *CMH1*, *MPD1*, Myosin Heavy Chain Slow Isoform
MYL2 Myosin, Light Chain 2, Regulatory, Cardiac, Slow	Cardiac Ventricular Myosin Light Chain 2, *MLC-2v*, *MLC2*
MYL7 Myosin, Light Chain 7, Regulatory	Myosin Regulatory Light Chain 2 Atrial Isoform, Myosin Light Chain 2a, *MYLC2A*, *MLC-2a*, *MYL2A*
NPPA Natriuretic Peptide A	*ANP*, *PND*, *ATFB6*, *CDP*, Natriuretic Peptide Precursor A, Cardiodilatin-Related Peptide
RYR2 Ryanodine Receptor 2 (Cardiac)	*ARVD2*, Cardia Muscle Ryanodine Receptor-Calcium Release Channel, Type 2 Ryanodine Receptor, *RYR*, *VTSIP*
TNNI1 Troponin I Type 1 (Skeletal, Slow)	Troponin I Slow-Twitch Isoform, *SSTNI*, *TNN1*
TNNI3 Troponin I Type 3 (Cardiac)	*CMD2A*, *CTnI*, Cardiac Troponin I, *TNNC1*, *CMD1FF*

(*Continued*)

Table 5.1 *(Continued)*

Gene Name	Synonyms
TNNT2 Troponin T Type 2 (Cardiac)	*CMH2*, *CMD1D*, Cardiac Troponin T2, *TnTC*, *cTnT*
TTN Titin	*CMD1G*, Connectin, *TMD*, *LGMD2J*, *EOMFC*, *MYLK5*, *MPRM*
VCL Vinculin	Metavinculin, *CMD1W*, *CMH15*, *MVCL*

*Current human gene symbols and associated synonyms/alias from the www.genecards.org database. Note: the list of synonyms is not comprehensive, but lists only a sampling of alternate gene names.

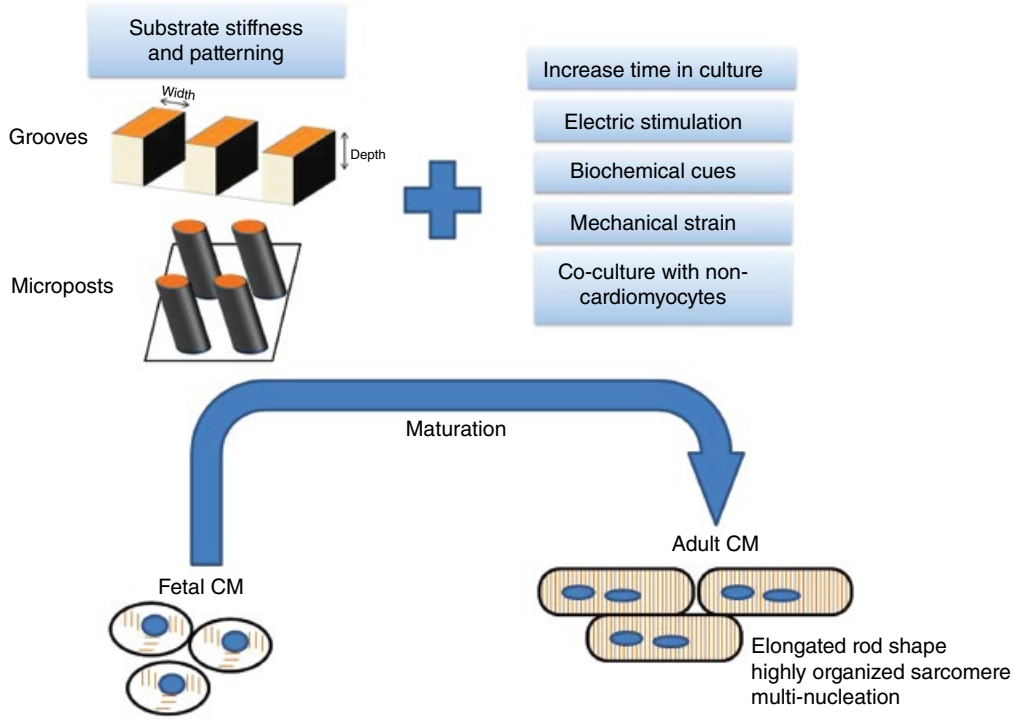

Figure 5.1 ***(Plate 1)*** *Examples of substrate patterns that have been used in an attempt to develop more mature cardiomyocytes. Various patterns in the substrate have been used alone or in conjunction with conditions listed on the right to produce a cardiomyocyte with a phenotype that is more similar to that of an adult cardiomyocyte. (See insert for color representation of the figure.)*

with additional cell types or including biochemical cues (Fig. 5.1/Plate 1; Yang et al., 2014; Veerman et al., 2015). Culture systems have either been two-dimensional (2D) or three-dimensional (3D), and some authors have developed tissue engineered "heart-on-a-chip" models (Heidi Au et al., 2009; Agarwal et al., 2013). The current review will focus primarily on alterations in the substrate that have been reported to affect the phenotype of the cultured cardiomyocytes. Much of the work to this point has been performed using neonatal rat cardiomyocytes, but studies using human cells will also be reviewed.

5.2 Lengthening Culture Time

Several laboratories have investigated whether increasing the length of time in culture could influence the maturation status of hiPSC- and hESC-derived cardiomyocytes. Kamakura et al. (2013) using a hiPSC line differentiated and maintained cells for up one year on fibronectin-coated culture dishes. After 2 years in culture, they observed a more mature sarcomeric structure, larger cells, a lower beating rate, enhanced expression of cardiac specific genes, and an increased number of cells that expressed the ventricular form of the myosin light chain 2 isoform (MYL2; Table 5.1) but reduced levels of the atrial isoform (MYL7). All of these are characteristic features of a more mature cardiomyocyte phenotype; however, the extended culture period diminishes the utility of this method.

Polyethyleneimine-gelatin coated coverslips were used to culture hESC-derived cardiomyocytes for 20–40 days or 80–120 days to compare maturation status (Lundy et al., 2013). The later stage cells structurally appeared to be more mature with about 33% of the cells having multiple nuclei, and all were longer with organized sarcomeres. These cells also contained a greater number of mitochondria, and the mitochondria contained more prominent cristae. The hESC-derived cardiomyocytes cultured for the longer time period also had an increased upstroke velocity, slower beating rate, and more mature calcium handling kinetics.

In a more recent study, Zhang et al. (2015) compared 2D and 3D formats for culture and maturation of cardiomyocytes differentiated from various hiPSC lines. For 2D culture, cells were cultured in a monolayer on Matrigel-coated plates. Three-dimensional cultures were initiated through the formation of embryoid bodies (EBs) in V-bottomed plates overnight; the EBs were transferred the next day to ultra-low attachment U-bottomed plates for continued maturation. Both culture formats gave rise to beating cells by six days of culture. Fluorescence activated cell sorting of various differentiated hiPSC lines indicated about 50–75% of the cells were cardiomyocytes in 3D culture; this was increased to 80–95% cardiomyocytes in 2D culture. Global gene expression indicated more variability in cell types present in the 3D cultures which helped to confirm the decreased purity of the cardiomyocytes in the 3D culture system. The 3D system did result in an overall greater number of cardiomyocytes; however, methods to remove non-cardiac cells would be needed in order to use this system in future applications. The transcriptome of cells cultured under both conditions became more stable after 4 weeks of culture. With time in culture, expression of pluripotent genes, cell cycle and proliferation genes decreased, and later developing cardiac genes were expressed. The authors concluded that their differentiation protocol could benefit from future developments in cell culture media that could enhance or accelerate development of more mature cardiomyocytes.

In another recent study, cardiomyocytes from both hESC and hiPSC lines were differentiated as EBs for 10 days prior to culture as a monolayer on 0.1% gelatin (Ribeiro et al., 2015). On day 13, some of the cells were transferred to cardiomyocyte media. After another 10 days of culture, some cells were transferred to polyacrylamide hydrogels that had been micro-contact printed with 20 μm wide 1% gelatin lines that were 20 μm apart. The hydrogels had a Young's modulus of 5.8 kPa. A group of cells continued in the cardiomyocyte media until day 33, while another group was transferred at day 20 to myocyte maturation media which contained the triiodothyronine (T3) thyroid hormone. Both the cardiomyocyte and myocyte maturation media were commercially available, serum-free and nutrient rich media. Thyroid hormone is known to be important for several facets of cardiac development including conversion of the myosin heavy chain, α form (MYH6) to the myosin heavy chain, β isoform (MYH7) and the conversion of titin (TTN)from the fetal to the adult form as well as several structural aspects of cardiac maturation (Veerman et al., 2015). Ribeiro et al. (2015) compared the contraction force of the cultured cells to that of cardiomyocytes obtained from 14, 17, and 19 week old fetuses that had undergone an elective abortion. The contraction force of the fetal heart cells was similar to each other suggesting little change in this feature between 14 and 19 weeks of gestation. The authors also observed that the contraction force of cardiomyocytes derived from hESCs and from hiPSCs were similar and significantly less than that of the fetal heart cells at culture day 23. Continued culture of the

hESCs and hiPSCs in cardiomyocyte media increased the contraction force and the surface area of the cells; the contraction force at day 33 in cells grown in cardiomyocyte medium was similar to that of the fetal cells. The cells grown in cardiomyocyte media also demonstrated a more mature electrophysiological phenotype with longer action potential duration and increased action potential amplitude when compared to hESCs and hiPSCs cultured in the standard differentiation media; however, there was no difference in resting membrane potential or upstroke velocity. The cells grown in the cardiomyocyte media were larger and also demonstrated a greater level of sarcomere organization. Continued culture in the myocyte maturation media led to a greater contraction force, further increases in sarcomere organization, action potential amplitude, and upstroke velocity with a decrease in resting membrane potential. Increased expression of several cardiac specific genes such as *MYH6*, *KCNJ12* that encodes the inwardly rectifying subfamily J member 12 potassium channel, as well as the cardiac ryanodine receptor 2 (*RYR2*) and the calcium-ATPase transporter, *ATP2A2*. Taken together, increases in contraction force, electrophysiological parameters, sarcomere structure, and gene expression all demonstrate a more mature cardiomyocyte phenotype in cells cultured in myocyte maturation medium indicating that in conjunction with the length of time the environment/media also influences the maturation status of hESC- and hiPSC-derived cardiomyocytes.

5.3 Substrate Stiffness

Several publications have examined the stiffness of the substrate as a physical cue to enhance cardiomyocyte differentiation. To allow comparison to the *in vivo* condition, the rigidity of the neonatal rat heart was found to be 4.0–11.4 kPa, while the adult rat myocardium was 11.9–46.2 kPa (Bhana et al., 2010). Other estimates of rigidity include approximately 70 kPa (Boublik et al., 2005) for adult rat heart, 54 ± 8 kPa for the right ventricle (Engelmayr et al., 2008) and 18 ± 2 kPa for the left ventricle (Berry et al., 2006). Jacot et al. (2010) measured the elasticity of left ventricles from embryonic and postnatal mice using atomic force microscopy indentation; they found that embryonic ventricles had elastic modulus of 12 ± 4 kPa, which increased to 39 ± 7 kPa after birth.

Polyacrylamide-based hydrogels have been used as substrates to induce the differentiation of mouse induced pluripotent stem cells (Macri-Pellizzeri et al., 2015). Hydrogels of various stiffnesses were coated with either collagen I or plasma fibronectin prior to placing an EB on the substrate. Polystyrene tissue culture plates coated with either collagen I or plasma fibronectin were used as controls. Three levels of stiffness were developed; these were approximately 0.6, 14, and 50 kPa. Gene expression markers were used to determine differentiation. Four cardiac markers were evaluated, *Myh6*, cardiac troponin T type 2 (*Tnnt2*), cardiac muscle 1 α actub (*Actc1*), and the calcium channel voltage-dependent L-type α1C subunit (*Cacna1c*); expression of the four markers was increased on all hydrogel stiffnesses and with either fibronectin or collagen I coating. Expression was slightly increased on the soft hydrogel coated with fibronectin when compared to collagen I coating, but expression of all markers was greater on hydrogels than on tissue culture plates. Morphologically, 70–80% of the clusters contained spontaneously beating cells, and transmission electron microscopy confirmed the formation of sarcomeres with visible Z-lines as well as the presence of abundant large mitochondria in the cells on 0.6 kPa hydrogel compared to the coated tissue culture plates. Also where cardiomyocytes were adjacent to one another, intercalated discs with desmosomes were present, suggesting cell-cell communication networks were established. The authors concluded that there was little difference in cardiac differentiation between the three stiffnesses of hydrogels, but most of their results compared only the softest hydrogel to cells cultured on tissue culture plates.

Ventricular myocytes isolated from postnatal day (PND) 1–3 rats have also been cultured on hydrogel with varying degrees of stiffness to determine if substrates contribute to cardiomyocyte maturation. Jacot et al. (2008) cultured isolated ventricular myocytes for seven days on collagen coated hydrogel coverslips; hydrogel

strengths included 1, 5, 10, 25, and 50 kPa. Cells on the stiffest substrate had a disorganized cytoskeleton with unaligned striations and no apparent sarcomeres; the cells cultured on the softest hydrogel also had very poorly defined striations. The cells cultured on 10 kPa hydrogel, however, had well developed and aligned striations. Western blots of ACTC1 and MYH6 expression did not show differences between cells grown on any gel stiffness. Calcium transients were greater in cells grown on 10 kPa gels than in cells grown on 1 or 50 kPa gels. The authors concluded that substrate stiffness does affect force generation, with maximal force generation observed in cells grown on substrates of 10 kPa, which is similar to the stiffness of native myocardium of neonatal rats.

In a similar, but more recent experiment, Bhana et al. (2015) also isolated cardiomyocytes from neonatal rats and cultured the cells on collagen-coated acrylamide hydrogels of varying stiffnesses. For control cultures, collagen-treated glass coverslips were used. Single cell suspensions were placed on the hydrogels and allowed to grow for five days. The cells on the stiffest hydrogel (144 kPa) and the control coverslips could not be induced to beat synchronously after an electrical stimulation was applied; cells grown on the softest hydrogel (3 kPa), however, were more easily stimulated to contract than were those cultured on a slightly stiffer matrix (50 kPa). The authors measured contractile force by displacement of fluorescently labeled beads in the hydrogels during contraction; they observed a decrease in the force of contraction of the cells with increasing stiffness of the gels. However, after 5 days of culture, the cells on the 50 kPa gels contained more cardiac troponin I (TNNI3) staining, longer cells and a higher aspect ratio (indicating more elongation and less spherical shape) than the cells grown on the lower concentrations of acrylamide. The authors concluded that substrates of about 50 kPa were optimal for culture of cardiomyocytes; this is very close to the range observed in native adult myocardium by these authors (11.9–46.2 kPa). These results differed from the findings reported by Jacot et al. (2008) who determined that 10 kPa was conducive to maximal force generation.

Cardiac cells isolated from embryonic Japanese quail, neonatal quail and chickens have also been cultured on collagen-coated hydrogels of various stiffnesses. The softer matrices (1 and 11 kPa) promoted the growth and striation of cardiomyocytes when compared to a stiffer matrix of 34 kPa. Additionally, on the softer matrices, the beat frequency of the cells was faster, and a greater percentage of the cells were beating (Engler et al., 2008).

Rather than acrylamide-based hydrogels, Galie et al. (2013) made polydimethylsiloxane (PDMS) substrates of 7, 27, 117, or 255 kPa; these were coated with laminin prior to culture of adult rat ventricular cardiomyocytes for 48 hours. Immunofluorescence was used to examine α-actinin 1 (ACTN1) and β1 integrin (ITGB1) organization within the cells. Although the amount of ACTN1 was the same across all of the substrates, it was organized into well-developed sarcomeres in cells grown on 7 or 255 kPa substrates but not in the cells grown on the 27 or 117 kPa substrates. Similar results were observed for ITGB1 presence within the cells. Expression of the genes for *Actn1* and *Itgb1* were increased in cells cultured on the 27 kPa substrate; α7-integrin (*Itga7*) and vinculin (*Vcl*) were elevated in cells cultured on both the 27 and 117 kPa substrates when compared to those on the 255 kPa substrates. Even though cells grown on the 7 and 255 kPa substrates demonstrated organized sarcomeres, there was a difference in their contractility. Cardiomyocytes grown on the 7 kPa substrate demonstrated increased shortening of the sarcomeres while cells grown on the stiffest substrate showed less shortening. The authors suggested that these data indicated that there was a range of substrate stiffness that was optimum for cardiac structure and function. However, the authors examined adult cardiomyocytes that were cultured for only 48 hours.

Copolymers of polyethylene glycol (PEG), poly-ε-caprolacton (PCL) and carboxylated PCL that differed in the ratios of the three components were fabricated by Chun et al. (2015) to examine the effects of substrate stiffness on cardiomyocyte differentiaiton. The copolymers were electrospun onto glass coverslips that were then coated with vitronectin. The authors found that a polymer of 4% PEG: 96% PCL produced the highest level of contractility and enhanced mitochondrial function in cardiomyocytes that had been differentiated from hiPSC. Cardiomyocytes cultured on the 4% PEG: 96% PCL polymer were the only cells to express high

levels of *Myl2*, a gene that is a marker of ventricular maturation that is present only in cardiac ventricles. Cells cultured on the other copolymers expressed primarily *Myl7*, which is characteristic of an immature cardiomyocyte phenotype and is expressed in both atria and ventricles. Additionally, cardiomyocytes cultured on the 4% PEG: 96% PCL polymer also produced TNNI3, which is present in more mature cardiomyoctyes; slow skeletal troponin I type 1 (TNNI1) is present in immature cardiomyocytes and was the primary tropoinin present in cardiomyocytes cultured on all other polymers. These results suggest that a polymer matrix consisting of PEG and PCL may allow more robust maturation of cardiomyocytes from hiPSC than other polymer matrices.

In an attempt to utilize information on characteristics of materials that enhance cell adhesion, Patel et al. (2015) used a combinatorial method to test more than 700 homo- and hetero-polymers that would allow enhanced maturation of cardiomyocytes differentiated from hESCs using either EB or monolayer differentiation methods. Contact printing was used to make microarrays on glass coated with poly(2-hydroxyethyl methacrylate) (pHEMA) to prevent background cell attachment. Their endpoints were cell attachment, size and sarcomere length (as determined by ACTN1 staining), and these endpoints were compared to cells cultured on 0.1% gelatin. Cells were grown in the absence of serum to rule out the role played by unknown serum constituents. A co-polymer of isobornyl methacrylate and *tert*-butylamino-ethyl methacrylate demonstrated the best differentiation and maturation of cardiomyocytes of all of the combinations tested. The authors also examined the electrophysiology of the cells by patch clamp methods. While the upstroke velocity was increased in cells grown on this substrate, the electrophysiology of the cells remained rather immature. The sensitivity of cells grown on this substrate to the cardiotoxic drug doxorubicin was also examined, and cells grown on the co-polymer were sensitive to disruption of myofibrils at a 10-fold lower concentration of doxorubicin when compared to cells grown on gelatin. These results suggested that this co-polymer substrate enhanced structural maturation of human cardiomyocytes; however, additional work is needed to produce more mature electrophysiology of the cells.

There are few studies that have examined the effect of substrate stiffness on cardiomyocyte maturation, and overall, results from these few studies are conflicting. These differences may be due in part on the cell type or cell line that was used as well as the culture media. However, it appears that substrates with Young's modulus of 50 kPa or greater generally do not support good cardiomyocyte differentiation. Combining substrate stiffness with some underlying structure added to the substrate may promote greater cardiomyocyte maturation.

5.4 Structured Substrates

Several authors have fabricated patterned substrates to determine if providing an underlying pattern would enhance maturation of cardiomyocytes. Wang et al. (2011) fabricated polystyrene or polyurethane substrates with 450 nm width ridges with 100 or 350 nm grooves between the ridges; these substrates were compared to flat surfaces of the same two materials. Neonatal rat cardiomyocytes were isolated and seeded onto the substrates. The cells aligned along the nanogrooves, although the alignment and maturation of the cellular phenotype (more elongated, formation of sarcomeres, formation of gap junctions) were greater with the deeper 350 nm grooves. Cell alignment was random on the smooth surfaces. There was little influence of substrate stiffness, in that there was little difference in cells grown on polyurethane or polystyrene even though there is a large difference in the elasticity of the two compounds; however, the cells cultured on the softer polyurethane retained the ability to contract for longer periods of time than did cells cultured on polystyrene.

Kim et al. (2010) fabricated a substrate from PEG hydrogels with ridges and grooves of various dimensions. Rat neonatal ventricular myocytes were cultured on the substrates. The cells aligned along the ridges and grooves versus a random orientation on the unpatterned substrate. The direction of contraction also

followed the patterned substrate as measured by fluorescent bead displacement. Conduction velocity was influenced by the substrate and followed the direction of the ridges and grooves. GJA1 expression was increased in the periphery of cells cultured on the patterned substrates, especially on the substrate with the widest ridges (800 nm wide and 800 nm between ridges) when compared with either the unpatterned substrate or the substrate with intermediate ridges (400 nm wide and 400 nm between ridges). The authors concluded that the patterned substrate altered the cell structure, which affected the functional aspects of the cell.

A different approach was taken by Rodriguez et al. (2011) in that rather than grooves, they placed microposts made from polydimethylsiloxane (PDMS) on glass coverslips that were first coated in PDMS. Fibronectin was added to the tips of the microposts to enhance the ability of the cells to attach to the posts. Rat neonatal cardiomyocytes were cultured on the substrate for five days prior to measurement of contraction. Although the cells were beating spontaneously, an electric stimulus was applied to assure contraction. The authors observed that the largest twitch forces occurred at the microposts at the perimeter of the cells, so all subsequent measurements occurred at the cell perimeters. As the micropost arrays became stiffer (3, 8, 10, and 15 kPa), the twitch forces were increased, but twitch velocity decreased. It appeared that the cells would twitch more slowly but more powerfully on the stiffer substrates. Although there were no differences in cell shape on the different substrates, sarcomere length and Z-band widths were increased on the stiffer substrates. Additionally, intracellular calcium concentrations and calcium transient during a contraction were higher on the stiffest substrate (15 kPa) compared to the softest substrate (3 kPa).

McDevitt et al. (2002) tested the morphology of cardiomyocytes grown on slides with laminin lines of various widths at various distances apart that were microcontact printed onto slides. Neonatal rat cardiomyocytes were isolated and cultured on the slides. The cells formed patterns along the length of the laminin. The elongated cells formed aligned myofibrils with desmosomes that allowed cell-cell communication. If the lanes were widely spaced, the cells beat synchronously along the lane; when the lanes were more closely spaced, the cells formed bridges that allowed cells from adjacent lanes to beat synchronously. The authors also observed that the laminin lanes could be contact printed onto biodegradable films; after several days of culture, the cells began to break down the substrate. This indicated that such substrates could be useful for tissue engineering applications.

LaNasa and Bryant (2009) used hydrogels with the proteins collagen I or laminin covalently bound within the hydrogel. They also tested an oligopeptide consisting of three amino acids, arginine-glycine-aspartic acid, which is commonly used for cell adhesion studies since it is present in a number of adhesive proteins. Unmodified hydrogels were used as one control, and wells coated with gelatin were used as a second control. Neonatal rat cardiomyocytes were seeded onto the hydrogels and cultured for 7 days. The cells did not attach well to the oligopeptide containing hydrogels, instead forming aggregates with little spreading. The cardiomyocytes did attach to the protein containing hydrogels; these cells also expressed TNNI3 and showed signs of striations. Gene expression was examined, and the cells attached to the protein modified hydrogels demonstrated low *Myh6/Myh7* expression; MYH7 is the predominant form in the fetal heart while MYH6 increases after birth. The low ratio in the cultured cardiomyocytes indicated that the cells did not develop a more mature phenotype, even though they showed signs of striations within the cells. ATP2A2 usually rises after birth, and expression of this gene was increased by day seven of culture in the protein modified hydrogels. A decrease in natriuretic peptide A (NPPA) expression is seen during maturation of cardiomyocytes, and expression of this gene was decreased in the cells cultured on the protein modified hydrogels. These data suggest that incorporating proteins into hydrogels enhanced cell attachment, and the cells demonstrated some signs of maturation during the culture period.

Neonatal rat ventricular myocytes have also been cultured on poly-(N-isopropylacrylamide) (PIPAAm) coated coverslips that were then treated with PDMS (Feinberg et al., 2012). Fibronectin was layered in a single layer for isotropic (ISO) conditions, or micropatterned in a way that added fibronectin in 20 μm wide ridges that were spaced 20 μm apart (LINES) or in an anisotropic pattern (ANISO). The cardiomyocytes

self-assembled based on the underlying pattern on the substrate. Electrical conductivity of the cells was measured, and the cells grown as LINES demonstrated longer action potential durations and faster propagation of action potentials than either of the other conditions. Cells grown on the ANISO conditions demonstrated conduction velocities that were similar to those of the LINES cells, but the action potential duration was longer in the LINES cells than in the ANISO cells. Calcium cycling was also faster in both ANISO and LINES cells compared to ISO cells, and the contractility of the cells increased with the increasing alignment of the cells. The authors concluded that the cellular architecture which was affected by the underlying substrate played a major role in developing the electrophysiological and contractility features of rat cardiomyocytes.

Neonatal rat cardiomyocytes have also been placed on an extracellular matrix derived from thinly sectioned (10 μm) decellularized material from adult hearts; cells grown on this matrix were compared to cells grown on plastic tissue culture dishes (Lee et al., 2015). Total cell number and cell viability were increased in cultures grown on extracellular matrix. The authors also examined gene expression for cardiac markers and found increased expression of *Tnnt2* and *Tnni3* in cells grown on extracellular matrix for seven or fourteen days; there was no change in GATA binding protein (*Gata4*) expression at either time point. Immunohistochemistry also indicated an increased level of ACTN1 in the cells grown on extracellular matrix for 7 days. These results suggested that a natural extracellular matrix could function to maintain the viability and proliferative capacity of neonatal rat cardiac cells while also maintaining the functional capacity of the cells. These decellularized matrices might also prove useful for transplantation studies.

Rao et al. (2013) developed a PDMS substrate with fibronectin-coated microgrooves 10 μm wide 10 μm apart and 4 μm deep in which human cardiomyocytes from Cellular Dynamics International (iCell Cardiomyocytes™) were cultured. As a control, cells were cultured on unstructured fibronectin-coated PDMS. Immunohistochemistry with an antibody to ACTN1 demonstrated that the microgrooved substrate led to a much more structured appearance of the sarcomeres than did the unstructured substrate. Calcium cycling was different between the two groups with cardiomyocytes cultured on the microgrooved substrate having a shorter transient amplitude under electrical stimulation; however, the reason for this difference in calcium cycling was not apparent. Expression of a number of genes was also analyzed; there were no differences in expression of the genes between cells grown on the structured or unstructured surfaces. In most cases, gene expression was much lower than that from a single human adult heart sample, and overall gene expression more closely aligned with that of a more immature cardiomyocyte. These results indicate that the structured substrate enhanced the structural phenotype and calcium handling of the human cells, but gene expression lagged behind these other effects. The authors concluded that more work needed to be done to develop substrates that would produce further maturation of the cells.

Gold coated glass slides with PDMS lanes of various widths patterned on the slides were developed by Salick et al. (2014) as a substrate for cardiomyocytes differentiated from hESCs. The slides were further coated in a mixture of fibronectin and Matrigel prior to seeding of purified cardiomyocytes onto the micropatterned slides. Lanes that were 30–80 μm wide produced cells with the most organized sarcomeres and aligned cell nuclei. GJA1 was expressed on both the lateral and axial edges of the cells, and there appeared to be no association between pattern width and GJA1 expression. There was also no association between calcium propagation rate and the width of the lanes, and the rate was slower than that observed *in vivo*. The authors concluded that the underlying geometry of the cell substrate presented important clues that could aid in differentiation of hESCs to cardiomyocytes.

Two different hESC lines and two hiPSC lines were cultured by Nunes et al. (2013) as biowires. The biowire is a device made of PDMS that contains a chamber with a sterile surgical suture. Differentiated dissociated cardiomyocytes that had been grown as EBs for 20, 34, or 40–44 days in media containing collagen and growth factor reduced Matrigel were seeded around the suture. A number of variables were tested in the biowires including cell size and shape, proliferation, distribution of cardiac proteins, development of sarcomeres,

conduction velocity, calcium handling, excitation threshold, hERG and I_{K1} channels, and expression of cardiac genes and proteins. Cells cultured in the biowires were aligned along the suture and contained well developed sarcomeres. These cells were more rod-shaped and were less proliferative than age-matched cells from EBs. Cells cultured in the biowires were also more mature in their electrophysiology and calcium handling than were cells from EB matched controls. Many of the features of these cardiomyocyte were intermediate between the fetal and adult phenotypes. A relatively small radius (300 μm) was chosen for the biowires since this size should allow diffusion of media without perfusion. The authors suggested that the addition of vascular cells may assist in improving survival and integration into host tissue in future *in vivo* regenerative studies.

Zhang et al. (2012) developed "matrix sandwiches" in which single cells from various hiPSC and hESC lines were cultured on a Matrigel-coated tissue culture plate and after a few days, the cells were overlaid with another layer of Matrigel making a matrix sandwich. Control cells were grown only on the lower layer of Matrigel. Various growth factors, including bone morphogenetic protein 4 (BMP4), basic fibroblast growth factor 2 (FGF2), and inhibin beta-A (INHBA), commonly known as activin A, were also added to the culture medium. By adding the upper layer of Matrigel, the cells formed multilayers and underwent an epithelial-mesenchymal transition as demonstrated by fluorescence microscopy of cells labeled with E-cadherin (CDH1) and N-cadherin (CDH2) and expression of genes associated with an epithelial-mesenchymal transition. By 30 days of culture, up to 98% of the cells were displaying TNNT2 and the presence of organized sarcomeres. The cell lines tested varied in the number of cardiomyocytes that developed from 40–92%; the culture method also worked to differentiate cells from cardiac disease models. Cells cultured for 15 days were compared to those cultured for 30 days to determine if the cells continued to mature. Expression of alpha smooth muscle actin (ACTA2), which occurs only in early cardiomyocyte differentiation decreased from day 15 to day 30; proliferative activity also decreased over this time frame. Electrophysiology of spontaneous contracting cells indicated that most of the action potentials observed in the cells were similar to those of embryonic cardiomyocytes than of more mature cells; however, they were similar to those of cardiomyocytes differentiated from EBs for 60–90 days. The authors concluded that although additional work is needed to induce further maturation of the cardiomyocytes, this culture method and defined culture conditions can be used to differentiate a variety of cardiomyocyte lines with easy scale-up of this matrix sandwich method.

Khan et al. (2015) cultured iCells cardiomyocytes on either gelatin coated tissue culture dishes or on nanofiber scaffolds coated with gelatin. The scaffolds were made of an FDA approved biodegradable substrate, polylactide-co-glycolide. A number of endpoints were evaluated, but the authors primarily focused on cellular morphology. The authors observed better alignment of the cells on the nanofibers with the development of sarcomeres. Cells grown on the nanofibers also had more mature mitochondria with denser cristae. The cells had faster calcium cycling, suggesting that they would be able to contract more rapidly, and synchronous beating of the cardiomyocytes was faster than that of cells cultured on the flat plates. Action potentials were similar among the cells grown on the two substrates. Additionally, ACTN1 and GJA1 were expressed in cells grown on both substrates, and there was little difference in gene expression of cardiac specific markers between cells on the different substrates. The authors concluded that the addition of a nanofiber scaffold led to cardiomyocytes that were structurally similar to those observed in normal heart tissue.

Lee et al. (2014) differentiated hESCs on one of three substrates: vitronectin coated graphene, vitronectin coated glass, or Matrigel. The cells were cultured over a period of 21 days. Vitronectin coating of the graphene enhanced cell attachment. The authors found that the cells cultured on coated graphene expressed more mesodermal and endodermal markers by 14 days of culture than did cells grown on the other two substrates; these cells also expressed greater levels of cardiac genes by culture day 21 than did cells on the other two substrates. However, the cells cultured on graphene were not beating spontaneously indicating that they had not fully differentiated to functional cardiomyocytes. The authors concluded that the nanoroughness of the

graphene aided in cell attachment and differentiation; however, it is unclear if continued culture would have led to more mature cardiomyocytes.

Zhang et al. (2013) used cardiomyocytes differentiated from a hESC line and then purified the cardiomyocytes by magnetic activated cell sorting. The purified cardiomyocytes were mixed in different ratios with non-cardiomyocyte cells that were in the original cell mixture; these cells expressed markers of smooth muscle cells, fibroblasts and endothelial cells. Final cell populations contained 48–90% cardiomyocytes in either 2D (monolayer) or 3D culture. A cardiac patch was generated using PDMS molds with elliptical pores that facilitated nutrient transport to the cells that were aligned around the pores. The cells were contained within a hydrogel solution that also contained fibrinogen, thrombin, and Matrigel. Under these conditions, the cardiomyocytes in the patch demonstrated increased expression of *TNNT2*, *MYH7*, the ventricular form of *MYL2*, calsequestrin 2 (*CASQ2*), and *ATP2A2*; expression of genes for electrical functioning of cardiac cells were not different between the monolayer and patch culture conditions. While conduction velocity was increased in the patches, action potential duration did not differ under these conditions. The cells in the 3D patch also had longer sarcomeres than did the monolayer cells. The authors also tested the ability of the cells in the patch to respond to the β adrenergic agonist, isoproterenol. The cells responded to increasing concentrations of isoproterenol with an increase force response. The authors concluded that the 3D cardiac patch enhanced maturation of the cardiomyocytes to a level similar to that of intact cardiac tissue.

hESC-derived cardiomyocytes grown either as EBs or as cells dissociated from EBs in a matrix of fibrinogen and thrombin was compared by Schaaf et al. (2011) to determine effects on cardiomyocyte maturation; the cell-fibrinogen-thrombin mixture was pipetted into molds with silicon posts present to form human engineered heart tissue. The engineered tissues demonstrated better sarcomeric development and organization. Additionally, those cells expressed the adult form of *MYH7*, but there were no differences in the other cardiac markers that were evaluated between the cell growth formats. However, the cells were more immature in their electrophysiology with longer action potential durations and very low maximal diastolic potentials. The authors concluded that although the cells did demonstrate some signs of increased maturation, they were still quite immature.

5.5 Conclusions

There are a number of endpoints to consider when determining if cardiomyocytes have reached a more mature phenotype. In some of the papers reviewed in this chapter, the structural phenotype appeared more mature than other endpoints such as electrophysiology or calcium handling. Longer term culture did enhance maturation of several features, but these longer culture times are often impractical for most applications. Patterning of the substrate also enhanced maturation of many of the structural aspects of the cells and this led, in several cases, to maturation of functional capabilities of the cells as well. However, the techniques needed to fabricate the substrates make these methods lower throughput. It may not be practical, or even necessary, to obtain fully mature cardiomyocytes for some applications, and compromises that allow higher throughput analysis may need to be made for other applications. It would seem that patterning the substrate in some fashion may be the simplest method to alter the structural phenotype of the cells, and functional maturation may follow.

Disclaimer

The opinions and conclusions expressed in this article are solely the views of the authors and do not necessarily represent the views of the U.S. Food and Drug Administration.

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6

Human Stem Cell-Derived Cardiomyocyte *In Vitro* Models for Cardiotoxicity Screening

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6.1 Introduction

6.1.1 Cardiotoxicity in Preclinical and Clinical Drug Development

Cardiotoxicity is a leading cause of attrition during both preclinical and clinical drug development. Drug-induced cardiotoxicity can be functional or structural in nature (as detailed further next), can occur with drugs both targeting cardiovascular and non-cardiovascular diseases and arise after acute or chronic drug treatment. Such late stage attrition is damaging in terms of the protection of patient safety and impacts the time-consuming, costly drug development process. Laverty et al. (2011) published the outcomes of a Medical Research Council (MRC) hosted cardiotoxicity workshop, which highlighted that preclinical screening strategies were effective at eliminating cardiovascular safety concerns prior to Phase I clinical trials (Laverty, et al. 2011). However, during late-stage development, when a greater number of patients were exposed, that cardiovascular adverse drug reactions were still an issue with implications spanning from prescribing restrictions to compound withdrawal.

Therefore, it is becoming apparent that highly sensitive, over-simplified, pre-clinical *in vitro* platforms may not be very specific in the detection of cardiac safety liability, and that there may be a lack of concordance

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of effects between preclinical species (*in vitro* or *in vivo*) and humans (Gintant, et al. 2016). Such failures demonstrate the demand for more translatable animal models and, where possible, the development of human-relevant paradigms that are more predictive much earlier in the drug discovery process. The development of predictive screening strategies that detect cardiotoxicity early in the drug discovery process will influence better decision making, the quality of drug candidates selected and reduce attrition, therefore, helping to ensure the development of safe medicines for patients.

6.1.2 Functional Cardiotoxicity

Functional cardiotoxicity encompasses effects on cardiac electrophysiology and cardiac contractility. Drug-induced disturbances in cardiac electrophysiology can lead to life-threatening arrhythmias, most notably prolongation of the QT interval and Torsades de Pointes (TdP), which is a major concern for drug companies and regulators (Laverty, et al. 2011). QT prolongation and/or TdP is associated with many cardiac and non-cardiac drugs including antihistamines, antiarrhythmics, antipsychotics, antibiotics and antimalarials. The most common cause for drug-induced QT prolongation is inhibition of the potassium channel hERG, which is responsible for the potassium current (IKr) involved in ventricular repolarization (Redfern, et al. 2003). A delay in ventricular repolarization through inhibition of hERG can result in reactivation of an inward depolarizing current (e.g. reopening of L-type calcium channels) known as early after depolarization (EADs). If these EADs are large enough in amplitude they may result in ectopic beats, which is a trigger for TdP. TdP itself can self-terminate, but it can also degenerate into ventricular fibrillation and cause sudden cardiac death. The International Conference on Harmonization (ICH) S7B guidelines detail the preclinical studies that are required by regulators to assess the potential risk of new drug candidates to delay ventricular repolarization, and, include an *in vitro* hERG assay and an *in vivo* non-rodent QT assay. However, compounds that inhibit hERG do not always prolong the QT interval or cause arrhythmias (Verapamil), compounds can prolong the QT interval through mechanisms other than hERG (Alfuzosin) and compounds that prolong the QT interval are not always proarrhythmic (Ranolazine). The Comprehensive *in vitro* Proarrhythmia Assay (CiPA) is a novel screening paradigm that has been proposed to replace the ICH S7B guidelines. Its focus is to move away from using hERG inhibition and QT prolongation as biomarkers/risk factors for proarrhythmia and adopt a more integrated mechanistic approach including multiple cardiac ion channel screening, *in silico* human ventricular cardiomyocyte modelling and confirmation of results in a human stem cell-derived ventricular cardiomyocyte assay (Sager, et al. 2014; Cavero and Holzgrefe 2015; Fermini, et al. 2016).

Current preclinical *in vitro*/*ex vivo* models employed to identify and assess the risk for drug-induced electrophysiological cardiotoxicity include cardiac ion channel screening and studies on isolated primary cardiomyocytes or Purkinje fibres, ventricular tissue preparations and whole hearts (Lawrence, et al. 2006; Pollard, et al. 2010). These models rely heavily on animals to make predictions on the clinical outcome, are low throughput, labour intensive, costly and may be unsuitable for routine early screening.

Cardiac contractility is an intrinsic property of heart muscle to produce contractile force and shorten, independent of heart rate, preload (sarcomere length) and afterload (pressure/load the heart has to contract against to eject blood), and is dependent on intra/extracellular calcium homeostasis. It is the process of excitation-contraction (EC) coupling that turns rhythmic electrical stimulation (action potentials) into mechanical force, allowing the heart to contract (Wohlfart and Noble 1982; Drake-Holland and Noble 1983). Depolarization of the cardiomyocyte membrane potential during an action potential results in Ca^{2+} influx into the cell through L-type Ca^{2+} channels, which in turn triggers the rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR) (Fabiato 1969; Fabiato, et al. 1971; Fabiato and Fabiato 1975). This increase in intracellular Ca^{2+} concentration activates myofilaments causing the cells to contract. Transfer of Ca^{2+} from the cytosol to the SR via the SR Ca^{2+} ATPase (SERCA) or through the Na^{+}-Ca^{2+} exchange (NCX), decreases intracellular calcium and results in cardiomyocyte muscle relaxation.

Changes in cardiomyocyte calcium handling, myofilament sensitivity to calcium and effects on the myocardial contractile machinery can all affect contractility.

Drug-induced changes in cardiac contractility are a safety concern and changes in cardiac contractility are investigated clinically by assessing changes in left ventricular ejection fraction (LVEF). Drug-induced increases in cardiac contractility (positive inotropy) places long term stress on the heart and drug-induced decreases in cardiac contractility (negative inotropy) lead to reduced perfusion of end-organs, both of which can lead to heart failure and increased mortality. Current preclinical models used to screen for drug-induced effects on contractility include ion channel and receptor screening, studies on isolated primary cardiomyocytes and whole hearts, *in vivo* telemetered studies and echocardiology measurements (Harmer, et al. 2012). These models have a heavy reliance on animals and measurements are often invasive, indirect, resource/time consuming and low throughput.

6.1.3 Structural Cardiotoxicity

Structural cardiotoxicity results in morphological damage or loss of cellular/subcellular components of the heart. It is associated with changes in multiple cardiac cell types leading to compensatory hypertrophy that may show ventricular-dilation, cardiac fibrosis (which interferes with cardiac compliance and contraction/relaxation), ultimately resulting in heart failure (HF) (Cross, et al. 2015). Cardiac cell injury progresses from degeneration, necrosis, inflammatory changes and fibrosis. Non-lethal cardiomyocyte injury can be characterized by vacuolation of the cardiac myocytes which may result from lipid accumulation, mitochondrial swelling or dilatation of the sarcoplasmic reticulum. The limited regenerative capacity of the heart suggests that adaptive responses to such degenerative insults may result in a predisposition to cardiotoxicity. Lethal cardiomyocyte injury results in necrosis, loss of membrane integrity and the release of cytosolic proteins. The morphological change includes an inflammatory response that may be accompanied by fibrosis and influence cardiac function (Cross, et al. 2015).

Structural cardiotoxicity may arise as a result of ‘on-target’ or ‘off target’ effects. An understanding of the potential ‘on target’ pharmacological effects may be raised as a result of an early, target biology review. As well as gaining a deep understanding of the target biology, this would also include assessing any human disease states or transgenic animal models, which may highlight a direct involvement of the desired target in cardiac structure and/or function. Harder to predict are the ‘off target’ pharmacological activities resulting from compound interactions with target(s) unrelated to the primary site of action.

Structural cardiotoxicity is associated with several chemotherapeutic agents, such as the anthracyclines (e.g. Doxorubicin) and small molecule tyrosine kinase inhibitors (e.g. Sunitinib, Sorafenib), and can include a range of undesired effects, for example heart muscle injury with cardiomyopathy and HF, complications of coronary artery disease leading to myocardial ischaemia, arrhythmias, hypertension and thromboembolism (Cross, et al. 2015). The incidence is dependent on a number of factors including the administered dose, cumulative dose, pre-existing risk factors, and may occur immediately or after a delay of months to years post-treatment (Cross, et al. 2015). With the introduction of new treatments and increased cancer survival times the delayed cardiotoxicity of some anticancer treatments can become evident a long time after completion of treatment (Ewer and Ewer 2015). Reviews on the drug-induced toxicity of anthracyclines and possible mechanisms of action are available (Minotti, et al. 2004; Yeh and Bickford 2009; Volkova and Russell 2011; Pizzino, et al. 2014).

The detection of structural cardiotoxicity is currently not possible using *in silico* or *in vitro* systems. Therefore, it remains dependent on the findings from animal models often performed later in development, with the detection of a variety of endpoints, for example heart weight, pathology (light microscopy and electron microscopy), biomarkers (cardiac troponins, atrial natriuretic peptides) and gene expression changes. Whilst the maturation phenotype of hiPSC-CMs remains a concern, the generation of these cell types and

their longevity in culture has begun to impact *in vitro* platforms, which may be applicable for the assessment of structural cardiotoxicity earlier in the drug development process.

6.1.4 Requirement for Improved *In Vitro* Models to Predict Human Cardiotoxicity

There is clear value in the use of *in vitro* human cardiomyocyte models suitable for routine preclinical cardiotoxicity screening. An opportunity, that circumvents the use of limited human tissue availability, lies in the use of *in vitro*-derived human cardiomyocytes from pluripotent stem cells (hPSC-CMs) (embryonic [ESCs] or induced [iPSCs] pluripotent stem cells). The development and commercial availability of such cells provides possibilities to routinely screen for drug-induced cardiotoxicity in a human cardiomyocyte model, which may improve predictivity/translation to the clinic, whilst beneficially, significantly impacting the 3Rs framework for humane animal research (i.e. refinement, reduction, replacement) by reducing the number of animals used for *in vivo*/*ex vivo* studies. However, it is important to appropriately characterize, quality control and validate such cells before integration into cardiotoxicity screening strategies to enable better decision-making and quality compound selection. Various hPSC-CMs and technological platforms have been assessed to evaluate their suitability for the detection of drug-induced cardiotoxicity. This review focuses on the application of some of those platforms that can either currently be employed, or are likely to be applicable in the very near future, to drug discovery and preclinical development.

6.2 Overview of hPSC-Derived Cardiomyocytes

Traditionally, primary adult cardiac tissue and animal models are considered the gold-standard in pharmacological profiling, but are low in throughput and not always suitable for routine screening. Human cardiomyocytes derived from pluripotent sources (hPSC-CMs) have paved the way as a widely-available, unlimited source of human cells that resemble aspects of human biology and provide potential applications earlier in drug discovery and development.

Multiple efforts are underway to generate large genetically diverse global libraries of healthy and diseased iPSCs, such as the UK Biobank, Corriell Institute of Medical Research, WiCell, HiPSCi and EBiSC for research to reflect the diversity of drug responses (Anson, et al. 2011) and iPSC banks that represent homozygous HLA haplotypes for cell transplantation (Taylor, et al. 2012; Turner, et al. 2013). Preclinical testing on patient-derived iPSCs can potentially be used to identify specific patient populations who would respond favourably or adversely to a drug (Itzhaki, et al. 2011; Lan et al., 2013, Liang et al., 2013; Ma, et al. 2013).

PSCs have accelerated our understanding of the developmental cues towards mesoderm and cardiomyocyte differentiation, thus yielding high-purity cultures of human cardiomyocytes (Zandstra et al., 2003; Laflamme et al., 2007; Hattori et al., 2010; Van Hoof et al., 2010; Khan et al., 2013; Lundy et al., 2013). For the induction of cardiac lineage (mesoderm), various strategies were utilized *in vitro*; the traditional embryoid body (EB) formation via the hanging drop or suspension culture, co-culture with supporting visceral endothelial (END-2) cells (Mummery et al., 2012), treatment with stage-specific growth factors, direct reprogramming of fibroblasts with exogenous factors, such as GATA4, Tbx5, Mef2c and Badf60c (Takeuchi and Bruneau, 2009; Ieda et al., 2010; Addis et al., 2013, Nam et al., 2013), and most recently with the use of pharmacological inhibitors (Lian et al., 2013). EBs provide an excellent tool for understanding early development since cell interactions within EBs can mimic the normal course of embryogenesis. However, this method of mesoderm development lacks reproducibility and efficiency (consisting approx. ~5–10% of cardiomyocytes), thus making it ineffective for scale-up of cardiomyocytes for its use in the pharmaceutical sector. Later, monolayer and other directed differentiation protocols with enhanced mesodermal lineage differentiation

were developed to produce more homogenous (enriched) population of cardiomyocytes with or without antibiotic selection (Kattman et al., 2011; Lian et al., 2013) or with selection using lineage-restricted promoters (NKx2.5, MYH6 and MLC2v) that regulate the expression of fluorescent proteins. (Anderson et al., 2007; Huber et al., 2007; Elliott et al., 2011; Burridge et al., 2014). Cell surface receptors, such as SIRPA and VCAM1 have also been utilized to isolate hPSC-CMs (Dubois et al., 2011, Ponten et al., 2013). As PSC culture moved away from animal-derived cells and reagents (xeno-free environments) (Ludwig et al., 2006), under defined growth factor (or serum-free) conditions (Yao Shuyuan, 2006; Brons et al., 2007; Yang et al., 2008), it guided the way towards the development of hPSC-CMs for the purposes of cell therapy, organ printing and cardiotoxicity screening. The most efficient and reproducible strategies for generation of an unlimited supply of cardiomyocytes involve inhibition of different signalling pathways and specific activation of others in defined culture conditions (Mummery, et al. 2012). One of the first directed differentiation protocols, although relatively inefficient, has provided insight into improving differentiation efficiency (Passier, et al. 2005; Xu, et al. 2008). Many variations of EB and monolayer protocols have been developed ever since (reviewed in Mummery, et al. 2012). Nowadays, scientists have achieved increased efficiency of cardiomyocyte differentiation yielding 80–90% pure cardiomyocytes using defined growth factor conditions or pharmacological inhibitors (Yang Xiulan, et al. 2008; Leschik, et al. 2008; Xu, et al. 2008; Yang, Soonpaa et al. 2008; Willems, et al. 2009; Lian, et al. 2013). One of the most efficient protocols available utilizes a growth factor-defined and serum-free system that can yield 0.8–1.3 million cardiomyocytes per cm^2 after 14d without cell sorting or selection (Lian, et al. 2013). Although, PSC-CMs are the desirable product for cardiotoxicity screening it is noteworthy to mention the utilization of cardiac progenitor cells (CPCs) in medium- to high-throughput screens. The investigation of pathways, targets and small molecules involved in the proliferation of heart-resident CPCs and their differentiation into adult cells broadens insight into fundamental mechanisms which may be of interest for *in vivo* regenerative therapies (Drowley, et al. 2016).

Early studies provided electrophysiological evidence of cardiomyocytes generating spontaneous electric field and action potentials typical of mixed population of nodal-, atrial- and ventricular-like cells (Khan et al., 2013). Differentiated cardiomyocytes are normally assessed by light microscopy for spontaneous beating, thus easily detected in culture as beating EBs or beating areas in monolayer differentiation approaches (Muller, et al. 2000). EB aggregates can be dissociated and plated on extracellular matrices like gelatin to measure their action potential morphologies (i.e. nodal, atrial or ventricular subtypes) using patch clamp electrophysiology. Complimentary phenotypic characterization techniques include microarrays and real-time PCR, immunolabelling against late structural myofilament proteins, such as Troponins (measured by flow cytometry or microscopy) and sarcomeric organization using electron microscopy. Fluorescence *in situ* Hybridization (FISH) or Comparative Genomic Hybridization (CGH) is used to ensure karyotype stability of iPSC material and electrophysiological studies to exhibit the nature and maturity of the differentiated cell subtypes.

Differentiation efforts from PSCs so far, have yielded a mixture of atrial, pacemaker-like and ventricular cell types, that more closely resemble the foetal cardiac phenotype by multiple criteria (Willems, et al. 2009), such as contractile protein expression and myofibrillar structure (Lundy, et al. 2013), electrophysiology (Binah, et al. 2007; Zhang, et al. 2009), calcium handling (Dolnikov, et al. 2006; Binah, et al. 2007) and force generation (Dolnikov, et al. 2006; Kita-Matsuo, et al. 2009). Thus, huge interest is drawn towards the maturation of hPSC-CMs (extensively reviewed by Robertson, et al. 2013; Yang, et al. 2014). In brief, prolonged cell culture of both ESC- and hiPSC-CMs (80–120 days) resulted in maturation to a more adult-like cardiomyocyte phenotype shown by expression of RNA and of structural/contractile proteins (Babiarz, et al. 2012; Lundy, et al. 2013). Other maturation efforts involve overexpression of calsequestrin (CSQ) a Ca^{2+}-handling protein (Liu, Lieu et al. 2009) that was absent in PSC-CMs (Dolnikov, et al. 2006) or the use of T3 hormone (Lee, et al. 2010).

Nonetheless, despite their limitations in terms of maturity and heterogeneity, the literature evidence suggests that hPSC-CM provide a useful source of human-relevant cells for cardiotoxicity screening.

6.3 Human PSC-CM Models for Cardiotoxicity Investigations

hPSC-CMs can be cultured in a variety of formats, of ever increasing complexity, with the aim of improving the phenotype towards human adult cardiomyocytes. However, increasing *in vitro* complexity has limitations in terms of the applicability of the technologies available for assessing cardiomyocyte function and compound-induced effects. As the emerging hPSC-CMs are characterized and assessed using the equally rapidly developing technologies, it is inevitable that much overlap is seen between culture platforms, technologies and the endpoints assessed. The hPSC-CM models and endpoints for cardiotoxicity investigations have been grouped next in consideration of their primary application, that is electrophysiology, contractility and structural cardiotoxicity endpoints, with some unavoidable overlap (Table 6.1).

6.3.1 hPSC-CMs for the Assessment of Electrophysiological Cardiotoxicity

hPSC-CMs express the major cardiac ion channels found in adult cardiomyocytes and are electrically active demonstrated by the generation of action potentials, and may provide a useful integrated model to screen for drug-induced electrophysiological cardiotoxicities. hPSC-CMs have been investigated in multiple platforms as a model to detect drug-induced electrophysiological cardiotoxicity including manual and automated patch-clamping (Caspi, et al. 2009; Honda, et al. 2011; Ma, et al. 2011; Jonsson, et al. 2012; Qu, et al. 2013; Gibson, et al. 2014; Scheel, et al. 2014), multielectrode array (MEA) (Clements, et al. 2009; Braam, et al. 2010; Guo, et al. 2011; Harris, et al. 2013; Navarrete, et al. 2013; Nozaki, et al. 2014; Gilchrist, et al. 2015; Kitaguchi, et al. 2015; Qu and Vargas 2015), impedance (Guo, et al. 2011; Jonsson, et al. 2011; Xi, et al. 2011; Abassi, et al. 2012; Peters, et al. 2012; Guo, et al. 2013; Himmel 2013; Scott, et al. 2014; Peters, et al. 2015), intracellular calcium imaging (Cerignoli, et al. 2012; Sirenko, et al. 2013; Sirenko, et al. 2013; Lu and Anderson 2015; Pointon, et al. 2015), voltage-sensitive dyes (VSDs) (Warren, et al. 2010; Herron, et al. 2012; Lopez-Izquierdo, et al. 2014) and optogenetics, which will be discussed in the following section.

Table 6.1 *Illustration of the platforms reviewed, and the overlap of some technologies across electrophysiology, contractility and structural cardiotoxicity applications*

Stem cell platform complexity	Electrophysiology endpoints	Contractility endpoints	Structural cardiotoxicity
Singe cell	Patch clamp		
2D monolayer	Voltage sensitive dyes		High content screening
	Multi-electrode arrays		SeaHorse flux analysers
	Impedance assays	Impedance assays	Impedance assays
	Calcium imaging assays	Calcium imaging assays	
	Optogenetics		
Complex, 3D and/or co-cultures	Voltage sensitive dyes	Cardiomyocyte thin films	Cardiomyocyte spheroids
		Engineered heart tissues	

6.3.1.1 Patch Clamp Assays

An *in vitro* hPSC-CM action potential assay has been used to examine compound-induced effects on hPSC-CM electrophysiology for proarrhythmic risk assessment using the patch clamp technique. Action potentials recorded from ventricular-like hPSC-CMs have been validated using reference compounds that affect various phases of the cardiac action potential.

In hPSC-CMs, hERG/IKr blockers prolonged the action potential duration (APD90) at concentrations that inhibited hERG/IKr and in some cases induced early after depolarizations. Results were consistent across studies using hPSC-CMs from different sources (hESC-CMs and hiPSC-CMs) and data correlated with preclinical and clinical effects (Caspi, et al. 2009; Peng, et al. 2010; Honda, et al. 2011; Ma, et al. 2011; Jonsson, et al. 2012; Qu, et al. 2013; Gibson, et al. 2014). In some cases, hPSC-CMs offered higher sensitivity over conventional *in vitro/ex vivo* animal models (Peng, et al. 2010; Gibson, et al. 2014). For example, Terfenadine prolonged the APD in hPSC-CMs, but failed to prolong the APD in canine and porcine Purkinje fibres, and the QT interval in isolated rabbit hearts. This data shows that hPSC-CMs are a sensitive model for detecting ventricular repolarization delay by hERG/IKr block and supports the use of a hPSC-CM action potential assay for assessing the risk for drug-induced QT prolongation and proarrhythmic risk caused by hERG channel block.

A hPSC-CM action potential assay may also be useful for examining chronic effects of compounds that produce delayed effects on the QT interval e.g. delayed QT prolongation through inhibition of hERG channel trafficking. The hERG trafficking inhibitor Pentamidine was used to evaluate the potential of hiPSC-CMs to detect delayed QT prolongation (Gibson, Yue et al. 2014). Pentaminide had no immediate effect on action potentials, but after chronic treatment for 20–24 h the action potential was prolonged and early after depolarizations were evident – compared to time matched controls – at therapeutically relevant concentrations. hPSC-CMs may be a useful model to study delayed/chronic effects on QT, but more compounds need to be studied to validate this fully.

The cardiac Na^+ channel (Nav1.5) is responsible for the upstroke of the cardiac action potential and depolarization. Block of Nav1.5 slows the upstroke of the cardiac action potential, prolongs the QRS interval on the ECG and slows conduction in the heart, which can lead to conduction disturbances and lethal arrhythmias. The effect of Na^+ blockers on action potentials recorded from hPSC-CMs has been tested to evaluate their potential to detect Na^+ channel depolarization liabilities. Differences in the sensitivity to Na^+ channel blockers in hPSC-CMs have been reported. Peng et al. (2010) showed that Lidocaine and Quinidine decreased the action potential upstroke velocity (Vmax) in action potentials recorded from hESC-CMs and were more sensitive compared to canine Purkinje fibres (Peng, Lacerda et al. 2010). In contrast, Qu et al. (2013) tested four Na^+ channel blockers (Flecainide, Mexiletine, AMG1 and Lamotrigine) in hESC-CMs and found that the cells were less sensitive at detecting Na^+ channel block compared to Nav1.5 ion channel data and QRS effects in an isolated rabbit heart assay (Qu, et al. 2013). The differences in sensitivity seen with Na^+ channel blockers could be down to different Na^+ channel blockers tested across studies and the maturation status of the cells. It is well known that hPSC-CMs are generally immature in their phenotype, based on various criteria (Willems et al., 2009). The cardiac action potential upstroke velocity (Vmax) is slow and the resting membrane potential is depolarized compared to adult human cardiomyocytes. The depolarized resting membrane potential may lead to a partially inactivated Na^+ channel population, which would result in a slower action potential upstroke. The immature phenotype may explain the differences in sensitivity to Na^+ channel blockers. For example, Lidocaine is use-dependent, preferentially binding to the inactivated state of the channel and may explain the higher potency of lidocaine in the hPSC-CM assay compared to Purkinje fibre assays (Wang et al., 2015).

In addition to hERG/IKr, IKs (KvLQT1/mink) contributes to phase 3 of the cardiac action potential. Block of IKs can prolong the cardiac action potential duration, which may be a risk factor for proarrhythmia.

There have been mixed reports of the effects of IKs block on the action potential of hPSC-CMs (Peng, et al. 2010; Honda, et al. 2011; Ma, et al. 2011; Jonsson, et al. 2012; Qu, et al. 2013). Qu et al. (2013) tested the effect of the IKs blocker L-766873, which did not prolong the action potential at concentrations that inhibited IKs, indicating that IKs was not present or active in these cells. Other studies tested the effect of another IKs blocker Chromanol-293B (Peng, et al. 2010; Honda, et al. 2011; Ma, et al. 2011; Jonsson, et al. 2012). In three studies, Chromanol-293B prolonged the action potential duration in both hESC-CMs and hiPSC-CMs (Peng, et al. 2010; Honda, et al. 2011; Jonsson, et al. 2012). Whereas another study by Ma et al. (2011) showed no effect of Chromanol-293B on action potentials recorded from hiPSC-CMs.

A hPSC-CM action potential assay has shown promise as a model to screen for drug-induced electrophysiological cardiotoxicities. However, larger compound screens including both true positives and true negatives are required to fully assess the predictivity of the hPSC-CM action potential assay, especially for detecting liabilities beyond hERG inhibition, for example Na^+ channel/depolarization and IKs liabilities.

While manual patch clamping is highly sensitive and produces high quality data, it is invasive, low-throughput as it is performed on single cells and labour intensive (requiring highly skilled staff in the technique). hPSC-CMs have started to be tested and validated on automated patch clamp systems to increase compound throughput and reduce resource (Ma, et al. 2011; Scheel, et al. 2014). Most commercially available automated systems are planar-based and use multi-well plates or chips, which enable recordings from multiple cells simultaneously. Cells are loaded into wells and suction is applied to pull the cells into the tiny holes in the plate to form a seal. The membrane is then ruptured or perforated to obtain the whole-cell configuration. The automated patch clamp systems that have the capability to operate in current clamp mode to measure cardiac action potentials include the CytoPatch 2 system (Cytocentrics) and the Patchliner system (Nanion). Both systems form GΩ seal enabling high quality recordings. Scheel et al. (2014) recorded action potentials from ventricular-like hiPSC-CMs (Cor.4U Axiogenesis) using the Cytopatch 2 and cells formed high resistant GΩ seals in this platform. Nifedipine decreased the action potential duration, Cisapride prolonged the action potential duration and TTX decreased the maximal slope of depolarization and shortened the action potential duration as expected. Results were comparable to those obtained from manual patch clamp recordings. These studies demonstrate that hPSC-CMs are compatible with automated patch clamp systems, providing an early high-throughput assay that could be used for routine screening of compound adverse effects on the action potential from hPSC-CMs. However, further validation with larger compound sets is required. Some considerations/limitations of automated patch clamp systems are the requirement for high purity cardiomyocytes to prevent contamination from non-myocyte cells since cells are captured blindly into the holes in the patch plate, voltage/current control can be lost in some platforms (e.g. to allow compound addition) and compounds may stick to the plastic surfaces of the plates in some systems, shifting dose-response curves to the right and decreasing sensitivity.

In addition to patch clamping, less-invasive/destructive technologies are available that utilize voltage-sensitive dyes or genetically encoded voltage and calcium indictors to optically measure action potentials from hPSC-CMs, and will be discussed next.

6.3.1.2 Voltage Sensitive Dyes (VSDs)

Voltage sensitive dyes (VSDs) are small molecules or proteins (e.g. Di-4-ANEPPS) that incorporate into the cell membrane and change their spectral/fluorescent properties in response to changes in the cell membrane potential, which can be visualized optically (Herron, et al. 2012). VSDs optically measure the electrical activity in the form of action potentials from single cells or monolayers of hPSC-CMs, allowing drug-induced effects on action potentials from hPSC-CMs to be assessed (Herron, et al. 2012). Electrophysiological endpoints that can be measured from the VSD-derived action potentials include: action potential duration (e.g. action potential duration at 90% depolarization), action potential upstroke rise time, triangularization,

proarrhythmic events such as early after depolarizations and spontaneous beat rate. Simultaneous recordings of action potentials from hiPSC-CMs using the patch clamp technique and VSDs demonstrated that there was correlation between action potentials recorded by the two techniques (Warren, et al. 2010; Lopez-Izquierdo, et al. 2014). The action potential durations recorded using VSDs were nearly identical to those measured directly using the patch clamp technique.

VSD platforms offer several advantages over conventional patch clamp action potential assays. VSDs provide increased throughput since experiments can be performed in multiwell plates (medium throughput assay), are less invasive, less labour intensive and reduce compound consumption, since compounds do not need to be continuously perfused. Cells can also be studied for several hours to days, allowing both acute and chronic compound effects to be studied, which is not possible with isolated cardiomyocytes or *ex vivo* heart preparations.

VSD platforms are being evaluated with hPSC-CMs as part of the CiPA initiative (Cavero and Holzgrefe 2015). A pilot study was carried out at four sites using hPSC-CMs from three different sources and four different platforms with a small compound set to evaluate the suitability of VSD recordings from hPSC-CMs. Preliminary data comparing the effects of three drugs on hPSC-CMs from the same supplier across two sites showed good correlation across sites. A larger compound validation study is currently underway.

Whilst VSDs offer advantages over conventional patch clamp techniques, there are also several limitations. VSDs can cause phototoxic damage to cardiomyocytes and readily bleach (photobleaching) resulting in signal run down, which limits recording time. The lack of continuous data recording means that compound effects cannot be monitored over time and it is not possible to know when steady state is reached. The VSD signals recorded can be small and may require averaging. High signal-noise ratio is essential and if the signal-to-noise ratio is low, small changes in the action potential will not be detected/will be missed. There may be motion artefacts in the signals caused by the cardiomyocyte contractions. The fluorescent readout is in arbitrary fluorescent units and absolute voltage measurements/readings cannot be obtained (cannot measure maximum diastolic membrane potential or upstroke velocity (V/S)).

6.3.1.3 Optogenetics

As the emphasis in safety pharmacology shifts to detection of the proarrhythmic event that triggers aberrant electrical conduction, novel techniques such as optogenetics are evolving to visualize the cellular action potential. As mentioned earlier, the manual patch clamp technique is labour intensive and low throughput and while voltage-sensitive fluorophores can resolve some of these issues, they can be associated with technical limitations cited previously.

In recent studies, researchers have described the expression of genetically-encoded voltage indicators, native to bioluminescent organisms, in mammalian cells as probes to visualize membrane excitability (Kralj, et al. 2012). Genetically encoded calcium indicators have also been used to probe the electrical behaviour of mammalian cells (Tian et al., 2012; Addis et al., 2013).

ArcLight, a variant of archaerhodopsin, was transduced in hESC-CMs and exhibited properties that paralleled the patch clamp detection of membrane potential (Leyton-Mange, et al. 2014). Not only did the fluorescence emission of the probe retain morphological characteristics of the myocyte action potential, but a linear shift in fluorescence compared to membrane potential was observed within the physiological voltages of the action potential (−60–20 mV) along with a rapid temporal response to voltage steps. In a neuronal preparation, the temporal resolution of a voltage spike was decreased to <500 μs, suggesting the ability to accurately capture an hPSC-CM action potential (Kralj, et al. 2012). Because this technique requires minimal to no manipulation of the cultured cardiomyocytes, serial measurements are possible to study maturation, differentiation and chronic drug-treatment. Since the CiPA initiative has proposed the use of multi-electrode array and/or voltage sensitive indicators to identify proarrhythmic potential, optogenetics has gained traction as a

possible platform to fulfil this goal. Studies suggest that compound-dependent prolongation of action potentials and early after depolarizations can be accurately identified using optogentic techniques in PSC-CMs (Leyton-Mange, et al. 2014; Shinnawi, et al. 2015), but it remains to be seen how this technology will compare to dye-based voltage sensitive indicators.

6.3.1.4 Multielectrode Array (MEA) Assays

Multielectrode array (MEA) technology is a non-invasive, label-free technique that has been used to study compound-induced effects on hPSC-CM electrophysiology. There are several commercial manufacturers of MEA systems including Multi Channel Systems, Axion BioSystems and MED64. Depending on the manufacturer, these commercially available MEA systems come in multiple formats with different plate types ranging from single well (low throughput) to multiwell plates (12, 48 and 96 wells, medium throughput). MEA uses microelectrodes embedded in the culture plate to measure extracellular field potentials (FP) from a monolayer of electrically coupled spontaneously beating hPSC-CMs. The FP waveform is composed of an initial depolarizing spike due to Na^+ influx, a plateau phase due to Ca^{2+} influx and a repolarizing wave/peak due to K^+ efflux (Halbach, et al. 2003). The initial depolarizing spike acts as a surrogate for the upstroke of the cardiac action potential and the QRS complex on the ECG (Halbach, et al. 2003). The field potential duration (FPD), measured from the initial spike to the peak of the repolarizing wave, correlates to the cardiac action potential duration and is a surrogate for the QT interval on the ECG (Halbach, et al. 2003). The FPD is rate dependent (longer at slower rates and shorter at higher rates). Given that hPSC-CMs spontaneously beat and compounds can induce changes in beat rate, FPD is often corrected for beat rate using Fredericia's or Bazett's correction formula (FPDc) (Batey and Doe, 2002). MEA can also detect arrhythmic events in hPSC-CMs such as early after depolarizations and ectopic beats.

hPSC-CMs have been pharmacologically validated using MEA to evaluate the ability to predict clinical drug-induced electrophysiological liabilities and arrhythmia (Caspi, et al. 2009; Braam, et al. 2010; Harris, et al. 2013; Navarrete, et al. 2013; Clements and Thomas 2014; Nozaki, et al. 2014; Gilchrist, et al. 2015; Kitaguchi, et al. 2015; Qu and Vargas 2015). Generally, compounds are tested acutely and endpoints assessed included FPD, FPDc, spike amplitude and slope, beat rate, early after depolarizations (EADs) and ectopic/arrhythmic beats.

Compounds that clinically prolong the QT interval and/or cause arrhythmias through inhibition of hERG/IKr (e.g. cisapride, terfenadine, dofetilde, quinidine etc.) prolonged the FPD/FPDc in hPSC-CMs at concentrations that inhibited hERG, and data correlated with preclinical and clinical effects (Caspi, et al. 2009; Braam, et al. 2010; Harris, et al. 2013; Navarrete, et al. 2013; Clements and Thomas 2014; Nozaki, et al. 2014; Gilchrist, et al. 2015; Kitaguchi, et al. 2015; Qu and Vargas 2015). Compounds that prolong the QT interval through mechanisms other than hERG/IKr inhibition have also been tested. The Na^+ channel activator Alfuzosin prolonged the FPD/FPDc in hESC-CMs and hiPSC-CMs (Navarrete, et al. 2013; Clements and Thomas 2014). Mixed effects of IKs inhibition on hPSC-CMs have been reported. The IKs blockers Chromanol 293B, JNJ282 and JNJ283 all prolonged the FPD/FPDc in hPSC-CMs (Clements and Thomas 2014; Nozaki, et al. 2014; Kitaguchi, et al. 2015). In contrast another study found that hiPSC-CMs were insensitive to the IKs blocker L768673 (Qu and Vargas 2015).

QT prolongation is a biomarker for proarrhythmia, but compounds that prolong the QT interval are not always proarrhythmic (e.g. Ranolazine). Early after depolarizations and ectopic/arrhythmic beats have been detected in MEA recordings from hPSC-CMs with proarrhythmic compounds. In addition to FPD/FPDc prolongation, EAD and ectopic beat detection may serve as important endpoints for predicting the proarrhythmic potential of compounds. Clements and Thomas (2014) investigated the effect of 21 compounds on MEA recordings from hESC-CMs, including compounds known to clinically prolong the QT interval and/or cause arrhythmias (Clements and Thomas 2014). To determine the value of a hESC-CM MEA assay in

predicting clinical TdP risk, a predicted risk score was generated for each compound by dividing the clinical efficacious concentration by the lowest concentration that induced a 20% change in FPD/FPDc or the highest concentration tested if ≤20% change was seen. They showed that anti-arrhythmic compounds or compounds removed from the market due to a high risk of Torsades (Redfern category 1 and 2) were ranked the highest, compounds known to prolong the QT interval but show low or no evidence of causing arrhythmias were ranked intermediately and compounds that do not prolong the QT interval or produce arrhythmias were ranked the lowest.

In another study, Qu and Vargas evaluated drug-induced FPD prolongation and early after depolarizations (EADs) as endpoints to predict clinical proarrhythmic risk (Qu and Vargas 2015). They compared the minimum effective concentration (MEC) that prolonged the FPD or induced EADs in hiPSC-CMs to the clinical therapeutic free concentration to create a ratio (MEC-FPD/Cmax or MEC-EAD/Cmax). The ratio was used to categorize compounds into low Tdp risk (ratio >30), intermediate TdP risk (>10 < 30) and high TdP risk (<10). The ratio for FPD and EADs had high sensitivity (high positive predictive power) but low specificity (low negative predictive power), and EADs were less sensitive than FPD for proarrhythmia prediction. For example, for FPD, a ratio of <10 for FPD incorrectly identified terodiline as TdP negative, and mexiletine and ranolazine as TdP positives. At ratios >30 FPD prolongation correctly identified all TdP positive compounds, but Ranolazine was still incorrectly labelled as TdP positive. This analysis suggests that hPSC-CMs are unable to distinguish between compounds with low and high TdP risk, which could generate a high number of false positives. However, one aspect lacking from this study was comparison to other preclinical models and the predictive value of these models to distinguish between compounds with low and high TdP risk.

As mentioned previously, block of Na^+ channels in the heart can lead to conduction disturbances and lethal arrhythmias. The initial spike in MEA recording from cardiomyocytes represents depolarization due to Na^+ influx (Halbach, et al. 2003). Inhibition of Na^+ channels in hPSC-CMs would be expected to decrease the spike amplitude and slope. The effect of Na^+ channel blockers on MEA recordings from hPSC-CMs have been tested to evaluate the spike amplitude and slope as endpoints to detect conduction disturbances driven by Na^+ channel block. Several studies have reported that Na^+ channels blockers decrease the initial spike and data correlated well with preclinical and clinical effects (Harris, et al. 2013; Clements and Thomas 2014). In a more detailed evaluation, Qu and Vagas tested the effect of several Na^+ channel blockers (selective and mixed ion channel blockers) in hiPSC-CMs and found no significant correlation between a compounds Nav1.5 IC50 and a decrease in spike amplitude and slope (Qu and Vargas 2015). A reduction in the spike amplitude and slope generally occurred at concentrations lower than the Nav1.5 IC50. In addition, there was discordance between a compounds Nav1.5 IC50 and its effect on the Na^+ spike/slope. The highly selective hERG channel blockers Dofetlide and Sertindole reduced the spike amplitude and slope at concentrations unrelated to Na^+ potency. For example, Dofetilide reduced spike amplitude and slope at concentrations ≥10,000x lower than its Nav1.5 potency. This discordance may be explained by the immature electrophysiological phenotype of hPSC-CMs. Action potentials recorded from hPSC-CMs display a depolarized maximum diastolic potential and phase 4 depolarization due to absence of the inwardly rectifying potassium current (IK1) (Peng, et al. 2010; Ma, et al. 2011; Gibson, et al. 2014). IK1 is important for setting the resting membrane potential and for the final stages of cardiac repolarization. In addition, hPSC-CMs may have reduced expression of IKs. Therefore, hPSC-CMs largely rely on IKr/hERG for repolarization and setting the maximum diastolic membrane potential. Compounds that inhibit IKr/hERG may result in a more depolarized diastolic membrane potential that would inactivate Na^+ channels and produce a decrease in the spike amplitude and slope. A larger evaluation is required to fully assess the spike and slope as endpoints to detect true Na^+ channel block and to avoid misclassifying compounds as having effects on depolarization.

Larger screens with true positive and true negatives compounds and comparison of data generated to current preclinical assays and clinical effects is required to fully assess the assays ability to predict clinical drug-induced electrophysiological cardiotoxicities and arrhythmia.

MEA is one of the platforms being evaluated with hPSC-CMs as part of the CiPA initiative (Cavero and Holzgrefe 2015; Fermini, et al. 2016). An initial pilot study was carried out at 12 sites using cells from four different sources and three different MEA platforms (Axion BioSystems, Multi-Channel Systems and ACEA Biosciences) to examine the effect of reference compounds on hPSC-CM electrophysiology (Cavero and Holzgrefe 2015). Eight compounds (Moxifloxacin, JNJ303, Quinidine, E4031, Flecainide, Ranolazine, Nifedipine and Mexiletine) were tested in triplicate at four concentrations/compound to examine sensitivity, reproducibility and predictivity across platforms, sites and stem cell sources.

There are many advantages of using hPSC-CMs with MEA technology. MEA provides non-invasive label-free measurements from a monolayer of electrically coupled cells, which is more physiological than single-cell invasive patch clamp measurements. MEA can be used to study both acute and chronic compound effects, which is not possible in conventional *ex vivo* isolated cell or tissue preparations, for example. the hERG trafficking inhibitor Pentamidine prolonged the FPD in hPSC-CMs over a 72 h period. MEA is higher throughput and less labour intensive than manual patch clamp assays, allowing more compounds to be screened in a shorter time frame. In some MEA systems it may be possible to image from MEA wells at the same time as making MEA recordings, allowing additional endpoints to be measured in a single experiment, for example calcium transient or contractility measurements. Limitations of MEA assays include cost since some plate types cannot be re-used. In addition, large amounts of data are generated that is time-consuming to analyse, making MEA assays unsuitable for high throughput screening and may limit continuous data recording. There are a number of semi-automated data analysis software programmes that have been developed to decrease analysis time (e.g. Notocord's Field Potential Screener and Neural IDs Intelligent Waveform Service). As the analysis software develops and evolves, data analysis time will decrease.

6.3.1.5 Impedance Assays

Cellular impedance platforms provide real-time, label-free, non-invasive measurements that use gold electrodes to measure changes in cellular impedance from a monolayer of spontaneously beating hPSC-CMs (Peters, et al. 2012; Peters, et al. 2015). Impedance works by applying a minute, slow alternating current through gold electrodes embedded in the bottom of special culture plates, which is impeded by the cell monolayer that covers the electrodes. The electrical current takes three paths; through cells (transcellular), in-between cells (paracellular) and around cells, providing a dynamic, sensitive measure for changes in morphology, cell-cell contact and adhesion. Impedance platforms can capture data at high speed allowing detection of cardiomyocyte beating through changes in the cell morphology as the cells contract. The impedance signal thereby acts as a surrogate/biomarker for cell contractility and impedance has been used to study drug-induced arrhythmia, effects on contractility and structural cardiotoxicity (Guo, et al. 2011; Jonsson, et al. 2011; Xi, et al. 2011; Abassi, et al. 2012; Peters, et al. 2012; Guo, et al. 2013; Himmel 2013; Scott, et al. 2014; Peters, et al. 2015). This section will focus on the application of impedance assays with hPSC-CMs for detecting drug-induced arrhythmias. The application of impedance for detecting drug-induced effects on contractility and structural cardiotoxicity will be covered in the stem cell models for contractility and structural cardiotoxicity sections, respectively.

Guo et al. (2011) tested the effect of 28 compounds with known cardiac liabilities on hiPSC-CMs using the xCELLigence RTCA Cardio system for assessing drug-induced arrhythmic risk. They compared the lowest compound concentration that produced ≥20% irregular beats (IB_{20}) to the clinical therapeutic concentration and found that compounds known to be proarrhythmic had IB_{20} values that lay within 10-fold or less of the clinical therapeutic concentration, whilst compounds that were not proarrhythmic had IB_{20} values that were >10-fold and above the clinical therapeutic concentration. The data from hiPSC-CMs on the RTCA Cardio system was found to be comparable to data generated from hiPSC-CMs using MEA technology (Guo, et al. 2011).

In a follow up study, further validation of the impedance assay was carried out using a set of 118 compounds, including reference and proprietary compounds (Guo, et al. 2013). For predicting clinical torsadagenic risk a threshold of ≤10μM for IB_{20} gave assay sensitivity, specificity and accuracy of 81% (identified 17/21 torsadogenic compounds), 82% (identified 42/51 non-arrhythmic compounds) and 81% respectively. Taking the clinical therapeutic concentration into consideration (using a threshold of ≤10-fold or less of the clinical concentration) slightly increased assay predictivity (81% sensitivity, 84% specificity and 83% accuracy). Inhibition of hERG and prolongation of the QT interval predicted clinical TdP with high sensitivity (identifying 18/21 (86%) and 21/21 (100%) torsadogenic compounds), however, the assays gave a high number of false positives (identifying 36/51 (71%) and 37/51 (73%) non-arrhythmic compounds respectively), resulting in overall assay predictivity of 75 and 81%, respectively. For predicting drug-induced non-TdP arrhythmia, using a threshold of ≤10 μM for IB_{20} or the clinical therapeutic concentration, 10/11 non-TdP arrhythmic compounds were identified (91% sensitivity). Inhibition of hERG and prolongation of the QT interval failed to predict drug-induced non-TdP arrhythmia, detecting 0/11 (0% sensitivity) and 4/11 (45% sensitivity) non-TdP arrhythmic compounds respectively. Compound-induced reductions in beat rate (BR_{20}, the lowest concentration that produces ≥20% decrease in beat rate) were used to predict QT prolongation. A threshold of ≤10 μM for BR_{20} gave assay sensitivity, specificity and accuracy of 88% (identified 35/40 QT prolonging compounds), 84% (identified 42/51 QT negative compounds) and 86%, respectively. Taking the clinical therapeutic concentration into consideration to predict QT risk increased assay predictivity (90% sensitivity, 84% specificity and 87% accuracy). In conclusion, Guo et al. showed that compound-induced irregular beats (IB_{20}) and decreases in beat rate (BR_{20}) were predictive of clinical arrhythmia (TdP and non-TdP) and QT prolongation respectively. IB_{20} was superior to QT prolongation and hERG inhibition in predicting drug-induced clinical arrhythmia. While cellular impedance can detect irregular beating indicative of arrhythmic events, it provides no information on the mechanism.

Advantages of hPSC-CM impedance assays for detecting drug-induced arrhythmia include a high throughput assay (up to 96-well format) that could be used for early hazard identification and compound de-risking. The platform provides real-time and non-invasive measurements so that compound effects can be studied over several days to weeks, allowing both acute and chronic compound effects to be studied. Limitations include cost as the impedance plates contain gold electrode that are non-reusable. In addition, larger amounts of data are generated that may limit recording time and may mean compound effects are missed.

Cellular impedance has been combined with MEA technology enabling the electrical activity in the form of field potentials to also be evaluated (Doerr, et al. 2015). The system has the advantage in that irregular beats detected in the impedance signal can be correlated with the underlying electrical activity thereby providing information on the mechanism. Combining multiple endpoints in one platform potentially reduces cost and captures compounds that may have been missed in electrophysiology experiments alone (e.g. blebbistatin). Cellular impedance combined with MEA is one of the systems being evaluated with hPSC-CMs as part of the CiPA initiative (Zhang et al., 2016). There are two commercial platforms available that combine impedance and field potential recordings: the cardio ECR (Cambridge Biosciences) and the CardioXcyte (Nanion). These platforms contain 1–4 MEA electrodes/well and if signal quality at these electrodes is poor or signals become immeasurable throughout the duration of the experiment then data will be unusable. The field potential and impedance electrodes are also located in different areas of the well, therefore measurements are from different areas of the monolayer so don't exactly match. The cardio ECR platform permits simultaneous measurement of impedance and FP, whilst the CardioXcyte platform can only make one measurement at a time. The technology is new and there is limited published data on these systems. The utility of these platforms with hPSC-CMs for cardiotoxicity testing is yet to be established.

6.3.1.6 Calcium Imaging Assays

The intracellular calcium signal couples cardiac electrophysiology (the cardiac action potential) with muscle contraction. Calcium transient measurements have been proposed to act as a surrogate for examining drug-induced effects on cardiac repolarization and proarrhythmic risk, since drug-induced changes in the action potential duration have been shown to result in drug-induced changes in the calcium transient duration.

Calcium transients can be visualized using calcium-sensitive fluorescent dyes (e.g. Fluo-4) and there are several commercial fluorescent imaging plate based platforms available that allow real time measurement of intracellular calcium transients (FLIPR and Kinetic Image Cytometer (KIC)). These calcium imaging systems are high throughput (96- or 384-well format), compatible with standard multi-well culture plates and compound handling robots, and could be used for early compound de-risking. The KIC system provides higher spatial temporal resolution compared to other plate-based readers used to measure calcium transients that measure from the whole well (e.g. FLIPR). KIC measures calcium transients from individual cells at hundreds of cells/well rather than measuring from the whole well, which eliminates the noise generated by the propagation of the signal across the field of view and/or asynchrony of the cells. In addition, the KIC system allows calcium transients to be measured from the whole cell or from a specific cellular compartment, for example the nucleus, cytoplasm and cell membrane. The calcium transients directly associated with release and reuptake of calcium from the sarcoplasmic reticulum can be analysed specifically by removing calcium transient signals from the nucleus.

Several publications have examined compound effects on calcium transients from hPSC-CMs as a model to screen for drug-induced electrophysiological cardiotoxicity (Cerignoli, et al. 2012; Sirenko, et al. 2013; Sirenko, et al. 2013; Lu, et al. 2015). In a recent study, Lu *et al.* (2015) performed a blinded validation study examining the effect of 53 compounds acting through a range of mechanisms (e.g. QT prolongers, QT shorteners, sodium channel blockers, calcium channel blockers, positive inotropic/chronotropic and negative controls) on calcium transients recorded from hiPSC-CMs using the KIC system (Lu, et al. 2015). They found that the most useful endpoints for identifying compound effects were measurements of the calcium transient duration, for example full width half maximal time (FWHM), decay time, time for the transient to decay from 75 to 25% (T75–25) and 75% duration of the calcium transient (CTD75). They showed that most compounds tested demonstrated the appropriate effect within the expected concentration range with the exception of Na^+ channel blockers that showed mixed responses. The system may not identify compound effects on sodium channels, since the calcium transient is initiated after membrane depolarization (action potential upstroke) when the sodium current is nearly complete. Calcium transient measurements from hiPSC-CMs may provide a useful high throughput screen for predicting drug-induced cardiac electrophysiological liabilities through a range of mechanisms for early compound de-risking. A wider compound set including a larger number of true negatives will need to be tested to allow assay predictivity to be assessed.

While measuring calcium transients from hPSC-CMs provides an early high throughput screen to predict drug-induced electrophysiological cardiotoxicity there are several limitations associated with using calcium-sensitive dyes. Like VSDs, calcium fluorescent dyes can cause phototoxic damage to the cardiomyocytes and readily bleach (photobleaching) resulting in signal run down, which limits recording time. In addition, calcium dyes may interfere with the biophysical properties of the cardiomyocytes, for example by sequestering internal calcium and affecting the spontaneous beat rate. The use of genetically encoded calcium indicators (e.g. Tnnt2-GCAMP5) would eliminate some of these issues since cells can be maintained and imaged in culture with time, as opposed to recorded as a terminal time point (Addis et al., 2013; Ifkovits et al., 2014).

6.3.2 hPSC-CMs for the Assessment of Contractile Cardiotoxicity

It has long been understood that culturing of embryonic stem cells can lead to the formation of beating aggregates or embryoid bodies (EB) that display contractile and electrophysiological properties of cardiac muscle (Doetschman, et al. 1985). Not surprisingly, the initial characteristic observed in the development of these

cells is the generation of rhythmic movement or contraction and has led to the advent of various platforms to replicate and measure contractility of the hPSC-CM-derived tissues, including muscular thin films and engineered heart tissues.

The impairment of cardiac pump function is what ultimately impacts vital organ perfusion and mortality. Therefore, understanding the strengths and weaknesses of *in vitro* contractility platforms is important in drug discovery and development. The isolated cardiomyocyte is a specialized cell that recapitulates the function of the intact heart, including the properties of excitation-contraction coupling, calcium homeostasis and signalling cascades that modulate cardiac contractility (Duthinh and Houser 1988; Harding, et al. 1991). Fractional shortening of cardiac myocytes has been used as a surrogate to understand pharmacological and pathological changes to cardiac contractility and is often compared to ejection fraction or force development of the intact organ.

6.3.2.1 Muscular Thin Films

The adult cardiac myocyte maintains a unique and organized morphology, normally referred to as 'rod-shaped' with a series of ladder-like structures comprised of myofilaments, which generate force in a linear direction. The muscular thin film (MTF) platform described by Feinberg et al. aligns neonatal rat ventricular myocytes and/or hPSC-CMs using micro-patterned extracellular matrix proteins as constraints on a biocompatible polydimethylsiloxane (PDMS), material for cells to adhere in a polarized pattern (Feinberg, et al. 2007). While varying the stiffness/elasticity of the PDMS can modulate the resting tension of the myocytes, active tension is generated by contraction and deflection of the substrate. Using standard video microscopy, the deflection of substrate is converted to force units by taking into consideration the elastic modulus and radius of contraction.

In situ studies exhibit resting sarcomere length of 1.8 μm and a length:width ratio of 7:1 for healthy mammalian cardiomyocytes (Onodera, et al. 1998). Culturing myocytes in this aligned pattern potentiates the deposition of myofilament proteins and enhances sarcomereogenesis, via the activation of focal adhesion kinase (Bray, et al. 2008). Observations from the MTF suggest that contractility increases the physiological aspect ratio, which is optimal for force generation of this two-dimensional preparation. Comparable measurements of peak systolic stress were measured from the MTF (9.2 +/− 3.5 kPa) and rat papillary muscle. Characteristic responses to pharmacological hypertrophic agents were also observed, suggesting the utility of this assay towards the identification of modulators of contractility. Additionally, the assay has been developed with increased throughput compared to dissociated primary myocytes and/or individual stem cell recordings. While structural constraints are important in influencing the morphology and ultrastructure of hPSC-CMs, mechanical and electrical stimuli can have an effect on myofilament deposition and organization, density of ionic currents that generate the action potential and electrical conduction.

6.3.2.2 Engineered Heart Tissues (EHTs)

Mechanical and electrical stimuli onto biocompatible materials in tissue engineering has facilitated the maturation of hPSC-CMs and automation/scalability of contractility measurements. Nunes et al. constructed a 3D cardiac tissue by culturing hPSC-CMs cells on a suture string embedded in type 1 collagen within a PDMS mould. These 'biowires' began to spontaneously beat within 2–3 days post culture, while cells became aligned along the axis of the suture string (Nunes, et al. 2013). To mimic the electrical conditions of the developing embryonic heart, the preparations were either stimulated up to 3 or 6 Hz for 1 week.

As evidenced by gene expression patterns of MYH6, atrial- and brain-naturetic peptide, culturing on the biowire substrate clearly promotes a decrease in expression of foetal genes, which is interpreted along with a decrease in stem cell proliferation, increase in cell size and expression of ion channel currents (IK1 and hERG)

as markers of maturation. Further, an increase in SR calcium load and positive chronotropic effect of epinephrine suggest an enhancement of excitation-contraction coupling. Culture of hPSC-CMs on the biowire platform ultimately led to an enhancement in the contractile apparatus, evidenced by well-defined Z-disks and myofibrilar organization. Stimulation at 3 and 6 Hz also led to increased deposition of desmosomes and ≈50% increase in electrical conduction. Ultimately, these culture conditions enhanced both the electrical (membrane potential and electrical capture of tissue) and mechanical (myofibril and gap junction) properties of the tissue, thereby producing a viable option for measurement of contractility (Xiao, et al. 2014).

Engineered heart tissues (EHTs) can be produced by mixing hPSC-CMs with a liquid hydrogel (e.g. fibrin, Matrigel, collagen), which is poured into a casting mould that determines the 3D structure of the tissue (Schaaf, et al. 2011; Hirt, et al. 2014; Stoehr, et al. 2014; Eder, et al. 2016). The hPSC-CM hydrogel mix attaches to a support structure, which provides mechanical restraint/load. These EHTs are force-producing 3D tissue constructs that provide a model to measure contractility. In a model described by Eder, et al. (2016), the hPSC-CM-containing hydrogel attaches to two flexible silicone posts that are positioned at either end of the casting mould in a standard 24-well plate format (Eder, et al. 2016). The gel block is removed from the cast and transferred to a cell culture plate where an aligned muscle strip forms and the hEHTs are ready to use after ~14 days. The hEHTs contract against the elastic silicone posts causing them to deflect. Force and contraction kinetics can be measured from the hEHTs by optical video recordings that measure the movement of the silicon posts. Deflection of the silicon posts (in mm) is used to calculate the contraction force (mN), taking into account the geometry and elastic properties of the silicone posts. The contractility endpoints that can be derived from this platform include force of contraction (peak force), time to peak (contraction time, T1 at 20% of peak height), time to relaxation (relaxation time, T2 at 20% of peak height), maximal contraction velocity (dF/dt) and maximal relaxation velocity (dF/dt).

Characterization of this hEHT model found a myocyte length-width ratio that was closer to an adult ventricular myocyte, but the cell size was still small (47.5 ± 2.1 pF in hEHTs vs 120 pF in adult human myocytes) although larger than published values for 2D hiPSC-CMs (18 pF). HPSC-CMs showed improved sacromeric organization and longitudinal alignment along the force lines compared to embryoid bodies or 2D hPSC-CM cultures, but the macroscopic organization was still less well developed than adult cardiomyocytes (Eder, et al. 2016). hEHTs express the gap junction marker Cx43, which is spread across the sarcolemma, whereas in the adult heart Cx43 is mainly confined to intercalated disks. Gene expression profiling provided some evidence of hEHTs promoting maturation, for example β-MHC (the adult isoform in humans) showed a time-dependent increase in expression that was not seen in EBs. However, sarcomeric proteins differed considerably from human ventricular myocardium. Maximal forces generated by hEHTS produced from hPSC-CMs ranged from 0.08–0.12 mN/mm^2, which is significantly lower than forces reached in intact heart muscles (40–80 mM/mm^2). The lower forces generated by hEHTs may be due to the smaller fraction of the hEHT occupied by cells and compact muscular strands compared to the whole heart, and the immaturity of the cells. External calcium concentration response curves revealed that hEHTs were hypersensitive to calcium, with a leftward shift of the force calcium relationship compared to adult hearts. Maximal force was reached at 2.2–3 mM external calcium in hEHTs produced from hESC-CMs and 5 mM external calcium in hEHTs produced from hiPSC-CMs. The force of contraction was therefore almost maximal in standard cell culture calcium concentrations and consequently there was an absent or low inotropic reserve. To detect the positive inotropic effects of compounds, experiments may need to be performed at submaximal external calcium concentrations (~Ca^{2+} EC_{50}), or else positive inotropic effects may be missed.

Hirt et al. (2014) subjected hEHTs to an electrical pacing protocol to induce cell maturation (Hirt, et al. 2014). Paced hEHTs produced higher forces and exhibited a better longitudinally aligned muscular network with a higher cytoplasm-to-nucleus ratio compared to unpaced hEHTs. Although hEHTs increase in maturity when chronically stimulated, they are still inferior to adult human cardiomyocytes.

hEHTs have been pharmacologically validated to assess their suitability as a model for cardiotoxicity testing (Schaaf, et al. 2011; Stoehr, et al. 2014; Eder, et al. 2016). The effects of positive and negative inotropes on contraction force and kinetics have been tested. Isoprenaline (tested at submaximal external calcium ~ the calcium EC_{50}) increased the force of contraction and showed a positive clinotropic (faster force development) and lusitropic (faster relaxation) effect as expected. The muscarinic agonist carbachol partially antagonized the effects of isoprenaline. Verapamil (tested at maximally effective external calcium concentrations) dose-dependently decreased the force of contraction in hEHTs. The proarrhythmic hERG channel blockers E-4031, quinidine, procainamide, sertindole and cisapride all decreased the relaxation velocity (T2 prolongaton) at published hERG IC50 values and at higher concentrations induced irregular beating patterns. The relaxation time may be a useful surrogate parameter of the action potential duration.

hEHTs have important advances over 2D hPSC-CM cultures. Different aspects of contractility can be measured in this model, including force and contraction kinetics, which are stable over several weeks. In addition, hEHTs contract auxotonically against the elastic resistance of the silicone posts, which is qualitatively comparable to the load imposed on the heart as it contracts against the pressure of the circulatory loading. This is more physiological than the isometric contractions in 2D hPSC-CM attached to non-physiological culture substrates. EHTs may also be useful in modelling cardiac diseases/pathophysiological conditions such as hypertrophy, since mechanical afterload can be increased by inserting metal braces into the silicon posts. With regard to toxicity testing, since hEHTs are stable in culture over several weeks, chronic compound treatments and repeated measurements should be possible, allowing delayed compound effects to be tested, for example hERG trafficking inhibitors or compounds that produce structural cardiotoxicity, which manifests clinically after extended periods. However, chronic or repeated applications are yet to be explored and compared to effects in 2D culture platforms.

The limitations of hEHTs are that they require large numbers of high purity hPSC-CMs for stable hEHT formation (e.g. 1×10^6 cells per EHT at >50% purity, around the equivalent of five wells of a 24-well plate when cultured as a monolayer), which makes the platform expensive compared to 2D systems especially when using commercially available cells. They take around fourteen days to form before use in experiments, whilst spheroids only take around 4 days to form. The development of co-culture models with cardiac fibroblasts and endothelial cells may be restricted due to the requirement for a pure hPSC-CM population. The platform is likely to remain low-medium throughput since miniaturizing hEHTs into 96-well format would impede manual handling steps and reduces the number of additional endpoints that can be studied, for example molecular, biochemical and histological. Currently, measurement of electrophysiology endpoints from hEHTs using sharp electrodes is technically demanding and invasive. Cells can be dissociated from the hEHT but this is also invasive and the dissociation process may damage and alter the cells. In the future, recordings could be performed using voltage-sensitive dyes or optogenetics. In addition, current measurements of calcium transients from hEHTs using calcium florescent dyes are inadequate since calcium-sensitive dyes (e.g. Fura-2) were shown to buffer intracellular calcium and change the force response to external calcium.

6.3.2.3 Impedance Assays

As mentioned previously, impedance acts as a surrogate/biomarker for cell contractility and has been used with hPSC-CMs to study drug-induced effects on contractility.

Scott et al. (2014) evaluated the effect of 49 compounds (30 inotropes and 19 non-inotropes) on the beating profiles of hiPSC-CMs using the xCELLigence Cardio System to validate the platform as a screening tool for identifying drug-induced changes in cardiac contractility (Scott, et al. 2014). These compounds had previously been tested *in vivo* and in a low throughput *in vitro* optical-based contractility assay that measured sarcomere shortening in electrically paced dog cardiomyocytes. Compared to *in vivo* contractility effects the hiPSC-CM impedance assay had sensitivity, specificity, and accuracy values of 90, 74 and 82%, respectively,

which was comparable to values reported for the dog cardiomyocyte assay (83, 84 and 82%). Potency data from hiPSC-CMs significantly correlated with data from the dog myocyte assay and lay within 5-fold of the dog myocyte data. While the impedance assay detected changes in activity in the impedance signal with inotropic compounds, the direction of the change was not always consistent and the assay failed to discriminate between positive and negative inotropes. The lack of discrimination between positive and negative inotropes may be a result of the immature phenotype of hiPSC-CMs. HPSC-CMs are morphologically round, small in size and have myofibrillar disorganization, and display foetal-like calcium handling, smaller forces of contraction and a negative force frequency relationship (Robertson, et al. 2013). In addition, measurements are obtained from 2D monolayers cultured on rigid non-physiological substrates, which only allows the cells to contract isometrically. Contraction in the adult heart is both isometric and isotonic. Whilst this platform has been evaluated and shown to be predictive for detecting compound-induced effects on cardiac contractility, it is unclear whether changes in the amplitude of the impedance signal really reflect changes in cell contractility, for example whether an increase in the amplitude reflects an increase in the force of contraction and a decrease in the amplitude reflects a decrease in the force of contraction. A hPSC-CM impendence assay could be used as an early screen to detect drug-induced effects on contractility and any hits could be followed up in an assay that provides a more direct mechanistic assessment of contractility.

6.3.2.4 Calcium Imaging Assays

The intracellular calcium signal couples cardiac electrophysiology (the cardiac action potential) to muscle contraction and, calcium transient measurements from hPSC-CMs have been used as a surrogate to study drug-induced effects on cardiac contractility. Pointon et al. (2015) investigated the effect of 51 compounds composed of 31 inotropes (22 negative inotropes and 9 positive inotrope) and 20 non-inotropes, on calcium transients recording from hiPSC-CMs using fluorescent Ca^{2+} imaging (FLIPR Tetra system) as an assay to detect drug-induced changes in contractility (Pointon, et al. 2015). The calcium transient endpoints measured included calcium transient number (peak count), transient amplitude, transient width, transient spacing and transient rise and decay time. Peak count was the most sensitive endpoint for detecting changes in contractility, correctly identifying 27/30 inotropes and 13/20 non-inotropes (7/20 non-inotropes were active), giving assay sensitivity and specificity in predicting the *in vivo* outcome of 87 and 70%, respectively. Effects on calcium transients by known inotropes mainly lay within 10-fold or less of the therapeutically relevant concentration. These results suggest that studying the effect of compounds on calcium transients recorded from hiPSC-CMs provides a useful early high throughput assay to screen for drug-induced changes in cardiac contractility. It should be noted that compounds that affect contractility through mechanisms targeting the contractile machinery would not be detected in this assay. Although the assay was able to detect changes in calcium transient activity with inotropic compounds, the direction of the change was not always also consistent and the assay could not discriminate between positive and negative inotropes. The lack of discrimination between positive and negative inotropes may be a result of the immature phenotype of hiPSC-CMs. Calcium handling in hPSC-CMs is comparable to foetal cardiomyocytes, with calcium transients displaying smaller amplitudes, slower kinetics and a negative force frequency relationship, which may be a result of hPSC-CMs lacking T-tubules and having reducing expression or lacking several proteins involved in calcium handling (Lee, et al. 2010; Robertson, et al. 2013; Li et al., 2013).

6.3.3 hPSC-CMs for the Assessment of Structural Cardiotoxicity

As previously mentioned, structural cardiotoxicity if often not detected until late in preclinical development, and there is no current consensus on an *in vitro* screening strategy. In part, this may be due to the lack of suitable predictive *in vitro* models (e.g. the inability to assess long-term effects) and mechanistic understanding

of structural cardiotoxicity. However, the emergence and availability of hPSC-CMs combined with the ability to simultaneously measure a range of parameters is beginning to cause a paradigm shift (Gintant, et al. 2016). In order to appreciate endpoints of potential relevance for investigating structural cardiotoxicity, it is pertinent to provide an overview of the mechanisms of cell death in a cardiomyocyte setting.

6.3.3.1 Mechanisms of Cardiomyocyte Cell Death as Endpoints in Drug Screening

The heart is an organ of limited regenerative capacity and excessive cardiomyocyte death is the primary reason for the manifestation of clinical symptoms (Fiedler, et al. 2014). It is important to pinpoint cell death signalling pathways in assay development read-outs for the profiling of drug responses in early drug discovery; which will be the focus of the current section.

The classical bifurfaction of cell death pathways are apoptosis and necrosis. Another category of cell death is autophagy, a complex orchestration of gene machinery that initiates an intracellular recycling process in response to the lack of nutrients, cellular stress and protein aggregation. Although autophagy can contribute to cardiac cell death in some circumstances (Pattingre, et al. 2005; Gustafsson and Gottlieb 2008b; Rothermel and Hill 2008), its role is not well understood. On the other hand, apoptosis and necrosis are widely accepted cell death modes that exhibit morphological differences underlined by mechanistically distinct, but convergent, signalling pathways. Thus, the term necroptosis is emerging. In the heart, cell death plays not only a pathophysiological role, but also contributes to the development of heart disease (Wencker, et al. 2003), therefore modulation of cell death can be a relevant therapeutic strategy. For this, it is worthwhile to introduce basic signalling pathways and how these can be manipulated to prevent cardiotoxicity using hPSC-CMs.

Apoptosis, an evolutionary conserved mechanism of programmed cell death (PCD) can be triggered by various stimuli that can activate intrinsic and/or extrinsic cell death pathways. Central to both pathways are caspase enzymes (from cysteine-dependent aspartate-specific proteases), which exist as pro-caspases (inactive zymogens) in the absence of a stimuli. The extrinsic (or death receptor, caspase-8/10-dependent) pathway results in the activation of cell surface receptors by binding of soluble death ligands (e.g. tumour necrosis factor-α [TNF-α]) or proteins bound to the cell surface of neighbouring cells (e.g. FasL), while the intrinsic (or Bcl-2-inhibitable, mitochondrial) pathway relies on an intermediary mitochondrial response through a variety of Bcl-2 family proteins of pro- or anti-apoptotic function. Pro-apoptotic proteins of the mitochondrial pathway such as Bax, Bak, Bid, Bam, Bim, Bmf, Noxa, Puma, Bnip3 and Bnip3L and anti-apoptotic Bcl-2 and Bcl-xL proteins also unite the extrinsic and intrinsic death signalling pathways (Chipuk et al., 2010), with a key convergence transducer protein known as Bid (BH3-interacting domain death agonist). tBid is cleaved by caspase-8/10, whereupon its C-terminal portion translocates into the outer mitochondrial membrane (Li et al., 1998). Activated Bid results in the allosteric activation of Bak (Wei, et al. 2000) that together with Bax form a conductance channel for the release of cytochrome-c (cyt-c) (Suzuki, et al. 2000; Crow, et al. 2004). The precise mechanism of release of other apoptogens and cyt-c is unknown, but a proposition is that interactions between Bim, Bad and Bax promote the opening of mitochondrial permeability transition pore (mPTP) (Shimizu, et al. 1999; Belzacq, et al. 2003; Cheng, et al. 2003) (Marzo et al. 1998). Cyt-c binds to apoptosis activation factor-1 (Apaf-1), which with ATP, recruits homo-oligomers of pro-caspase-9 via dimerization into an apoptosome complex (Li et al., 1997, Yuan and Akey, 2013). Assembly of the apoptososme complex results in activated caspase-9, which then can subsequently activate downstream effector caspase-3 (Acehan et al., 2002; Yu et al., 2005). The initiation of the extrinsic pathway via the death receptors by ligand binding induces the recruitment of the adaptor protein Fas-associated via death domain (FADD) and binding to pro-caspase-8 forming the death inducing signalling complex (DISC). Dimerization of pro-caspase-8 in the complex results in its self-activation (Oberst et al., 2010) and of subsequent activation of caspase-3 (Deveraux et al., 1998).

Apoptosis is held in check by 'mitochondrial gatekeepers' of the anti-apoptotic family (Bcl-2 and Bcl-xL) that potentially bind to Bax and Bak or proteins of the voltage-depended anion channel (VDAC) to prevent release of cyt-c (Yang, et al. 1997). However, Bcl-2 has a more general role in cardiomyocyte homeostasis and its survival effect is not limited to its anti-apoptotic function (Chen, et al. 2001), but in its ability to reduce acidification and ATP consumption, evident by a model of cardiac-specific overexpression of Bcl-2 that minimized ischaemia/reperfusion (I/R) injury (Imahashi, et al. 2004). Another potential inhibitor c-FLIP [FLICE-(FADD-Like IL-1β-converting enzyme)-inhibitory protein], enriched in striated muscle (Shu, et al. 1997), binds to pro-capase-8 to prevent DISC formation. Interestingly, c-FLIP exists in two isoforms, where the long isoform at low concentrations activates pro-caspase-8, but at high concentrations (Peter, 2004) has an inhibitory role (Whelan, et al. 2010). Inhibition of the extrinsic pathway by the short isoform of FLIP occurs by occupying FADD and pro-caspase-8 to preclude DISC complex (Whelan, et al. 2010).

Mammalian inhibitors of apoptosis (IAP), such as X-linked IAP (XIAP) also directly bind caspases to inhibit their catalytic activity (Huang et al., 2001). IAPs utilize their E3 ubiquitin/ligase activity for proteasome degradation of caspases as a safety mechanism for transient or accidental cyt-c leakage from eliciting a full apoptotic response (Deveraux et al., 1998). Mitochondrial apoptogens such Smac/DIABLO and Omi/HtrA2, released by sufficient activation of caspase-9, bind to IAPs (c-IAP1, c-IAP2, XIAP) to neutralize caspase inhibition (Du, et al. 2000). At the same time IAPs can bind pro-caspase-9 directly to inhibit apoptosome formation. XIAP can be irreversibly inactivated by the serine protease activity of Omi/HtrA2 (Verhagen, et al. 2000; Verhagen, et al. 2002). In contrast to their involvement with apoptosis, cIAPs also play a critical role in the regulation of cell death that occurs by death receptor activation (Whelan, et al. 2010).

Apoptosis Repressor protein with a CARD domain (ARC), which is expressed in cardiac and skeletal myocytes, exerts an inhibitory effect on both intrinsic and extrinsic pathways (Nam, et al. 2004). ARC engages with the death domains of Fas and FADD to inhibit DISC assembly, as well as antagonizing the intrinsic pathway by ARC-Bax complex formation that prevents Bax activation and translocation to the mitochondria (Nam, et al. 2004). ARC protein levels decrease in response to death stimuli in MI model (Nam, et al. 2007) and its knockdown triggers spontaneous Bax activation and apoptosis (Nam, et al. 2004).

Necrosis is the alternative pathway to apoptosis and is particularly important to MI and drug cardiotoxicity (Montaigne et al., 2012). The quintessential features of necrosis are the depletion of ATP and loss of plasma membrane integrity, both of which are nowadays interrogated in various cell-death based assays. There are two distinct, but at the same time convergent, branches of necrosis: the regulated death receptor signalling branch of TNF receptors and the mitochondrial permeability transition pore (mPTP) (Konstantinidis et al., 2012). Activation of death receptors via TNF signalling results in survival, proliferation or cell death. Treatment of cells with pro-inflammatory TNF cytokine alone does not promote cell death, since both survival and death mechanisms are activated (Whelan, Kaplinskiy et al. 2010). However, TNF can induce apoptosis in the case of inhibition of survival mechanisms, whereas TNF can elicit necrosis when caspases are inhibited (e.g. with a pan-caspase inhibitor) (Whelan, et al. 2010). TNF-induced cellular responses are mediated by either TNFR1 or TNFR2 – the two receptors of the TNF-receptor superfamily – the latter receptor mainly activated to achieve apoptosis or necrosis (Liu 2005). These dual effects may underlie the discouraging results of TNFα antagonists in early clinical trials (Feldman, et al. 2000; Mann 2005) for heart failure pushing towards the requirement of selective inhibitors against TNFR1 alone (Mann 2005). TNFR1 stimulates the formation of either of two complexes (complex I or complex II) with the recruitment of various adaptor proteins and serine/threonine kinase RIP1 (Micheau and Tschopp, 2003). Simply, complex I formation promotes cell survival whereas transition to Complex II results in cell death. Complex I comprises RIP1 kinase, TNF receptor-associated factor 2 (TRAF2), the adaptor TRADD protein and inhibitors of apoptosis c-IAP1 and -2. cIAP-1 and -2 ubiquitinate RIP1 kinase and TRAF2. Polyubiquitinated RIP1 and TRAF2 subsequently recruit and activate TGF-β-activated kinase-1 (TAK1) through TAK-1 binding protein (TAB2/3) (Wang et al., 2008). TAK1 kinase is a member of the MAPKKK cascade that phosphotylates NFkB (IkBα, IkBβ or IkBε)

and part of the IKK complex, elevating the block of NFkB nuclear translocation (Yu et al., 2008). The downstream effect is the activation of survival genes (Whelan, et al. 2010). In other settings, TAK1 can drive cardiomyocyte death (Zhang, et al. 2000). Interestingly, polyubiquitinated RIP1 binds via its NEMO polyubiquitin chains achieving recruitment of IKK (Devin, et al. 2001; Ea, et al. 2006). Mutations of NEMO were shown to abolish IKK activation (Ea, et al. 2006). Transition of complex I to II towards cell death signalling involves its dissociation from TNFR1 and cytosolic internalization. RIP1 is de-ubiquitinated by CYLD (cylindromatosis) and stimulates the recruitment of DISC complex, where caspase-8 targets RIP1 for C-terminal cleavage (Lin et al., 1999; Chan et al., 2003), thus obstructing RIP1 to signal necrosis or survival, but will stimulate apoptosis. However, in the case of pharmacological inhibition or genetic ablation of caspase-8, RIP3 is recruited into a complex with RIP1 (RIP1/3), where both are activated by phosphorylation (Lin, et al. 1999; He, et al. 2009). This molecular relationship between necrotic and apoptotic signals is reinforced by double-knockout mice of RIP3-caspase-8 and RIP1-FADD, where embryonic lethality caused by caspase-8 knockout and FADD deficiency is rescued by loss of RIP1 or RIP3 (Feoktistova, et al. 2011). It is worth noting that, so far, pathway dissection of TNFα-induced programmed necrosis was studied in inflammatory, cancer cells and fibroblasts. However, the role TNF cytokine in cardiomyocytes appears to be generation of apoptotic signals via caspase-8 receptor, which are diminished with Bcl-2 overexpression (Haudek, et al. 2007). In addition, a small molecule inhibitor called necrostatin-1 (nec-1) lead to reduced infarct size following I/R, independent of CypD and mPTP opening, via inhibition of RIP1 kinase activity. Therefore, hPSC-CMs offer an opportunity for further delineating the contribution of RIP1/RIP3 signalling axis in cardiomyocyte cardiomyocyte cell death.

The second branch of necrosis is the mPTP-dependent pathway, where mPTP is involved in the modification of permeability properties of the mitochondrial inner membrane and is a major pharmacological target for diseases such as I/R, heart failure and cardiotoxicity (Brenner and Moulin 2012). While the structure of mPTP is yet unidentified, it is a voltage-dependent anion channel for molecules up to 1.5 kDa regulated by adenine nucleotide (ADP/ATP) translocators (ANTs), cyclophilin D (CypD; protein, PPIF; gene) and members of the Bcl-2 family. In the context of I/R, high oxygen concentrations lead to increased oxygen uptake by the respiratory chain, thus yielding higher, than physiologic, levels of Reactive Oxygen Species (ROS) (Gustafsson and Gottlieb 2008a). The high density of mitochondria in cardiomyocytes leads to enhanced levels of superoxide, quickly converted into hydrogen peroxide, which fails detoxification by antioxidant systems (i.e. MnSOD, glutathione peroxidase) (Gustafsson and Gottlieb, 2008a). This results in oxidative modification of mitochondrial proteins and a series of molecular events, culminating in mitochondrial dysfunction and cell death (Gustafsson and Gottlieb 2008a). In summary, the generation of ROS can cause the collapse of $\Delta\Psi m$, action potential destabilization and opening of mPTP, which under normal conditions is tightly regulated to maintain pH gradient and membrane action potential. Decrease of ATP/ADP ratio leads to a drop in pH gradient in I/R, that eventually results in collapse of the proton gradient and opening of mPTP. The importance of this is demonstrated by pharmacological inhibition with mPTP inhibitor Cyclosporine A where CypD-deficient mice, whose cardiac mitochondria are resistant to mPTP opening in response to Ca^{2+} and oxidative stress, have smaller heart infarcts compared to WT littermates (Baines, et al. 2005; Baines 2007; Nakagawa et al., 2005). Interestingly, CypD-deficient mice (Baines, et al. 2005) remained sensitive to apoptotic stimuli, such as TNF-α and staurosporine. In a recent study by the Molkentin lab, it was shown that apoptotic regulator proteins of the Bcl-2 family, Bax and Bak, are required for mPTP-dependent cell death by facilitating the permeability of the outer mitochondrial membrane, but without affecting the inner membrane of mPTP function (Karch, Kwong et al. 2013). Among the Bcl-2 family, is the BNip subgroup (BNip1, BNip2, BNip3 and Nix/BNip3L), members of which can uniquely stimulate cell death with features of both apoptosis and necrosis (Yussman et al., 2002; Diwan et al., 2007). Nix/BNip3L (Nix) is responsible for cardiomyocyte cell death and heart failure after chronic pressure overload (Yussman, et al. 2002; Diwan, et al. 2007). Using endoplasmic reticulum (ER)- or mitochondrial-directed Nix mutants, it was

evident that Nix-mediated necrosis involves crosstalk between the mPTP-dependent mitochondrial pathway and ER, whereas mitochondrial Nix induces only caspase-dependent apoptosis (Chen, et al. 2010).

The crosstalk between ER and mitochondria of various proteins appearing to be central to both apoptosis and necrosis pathways is evidence that cell death is dependent on cell context and stimulus, and that pathways are not dichotomous platonic entities, but rather highly overlapping. The basic roles of ER are (i) to facilitate protein folding, phospholipid synthesis and glycosylation with the aid of calcium-dependent chaperones (e.g. Grp78, Grp94 and calreticulin) and (ii) to function as stores of intracellular Ca^{2+}, a critical messenger for muscle contraction (Xu et al., 2005; Minamino et al., 2010). Perturbation of these processes by compounds (e.g. dithiothreitol and SERCA inhibitors thapsigargin or tunicamycin and adriamycin) or deposition of β-amyloid aggregates (Pattison, et al. 2008) can elicit the unfolded protein response (UPR). Prolonged ER stress can feed forward into apoptotic and mitochondrial cell death via the ER calcium-release channel. Calcium overload of isolated mitochondria from cardiomyocytes has also been shown to mediate release of cyt-c (Logue, et al. 2005) and opening of mPTP from mitochondrial swelling due to depolarization of the inner mitochondrial membrane. Although the role of Ca^{2+} in mPTP opening is unclear, as most evidence arise from isolated mitochondria, it is highly important in apoptosis. Under ER stress conditions, pro-apoptotic transcription factor CCAAT/enhancer-binding homologous protein (CHOP) can function by mediating transcriptional induction of Bim and its translocation to the ER membrane and by repression of Bcl-2 and Bnip3 in cardiomyocytes (Minamino, et al. 2010). Human hypertrophic and failing hearts exhibited increased levels of GRP78 and CHOP, and failing mouse hearts appear with coincidental appearance of DNA fragmentation assessed by TUNEL staining. Treatment of isolated rat cardiomyocytes with pathological stimuli, such as SERCA inhibitors, TNFα and angiotensin II that can activate caspase-3 coincided with ER activation (Okada et al. 2004). Pro-apoptotic member or the Bcl-2 family, PUMA, was induced in mouse hearts in response to ER stress or treatment of neonatal rat cardiomyocytes with SERCA inhibitors. Treatment of cardiomyocytes with the inhibitors after knockdown of PUMA also attenuated ER-mediated cell death and release of cyt-c from mitochondria (Nickson, et al. 2007). Calpains, non-caspase Ca^{2+}-dependent proteases, are also important in ER-induced cell death, with conventional forms -m and -μ implicated in the cleavage of Bax and Bid proteins, leading to their subsequent activation (Chen, et al. 2001) and in the degradation of substrates such as PKCa, desmin and procaspases (Patterson, et al. 2011). Furthermore, treatment of nenonatal rat cardiomyocytes and mouse hearts with calpain inhibitors or overexpression of calpastatin (endogenous calpain inhibitor) in the presence of doxorubicin enhanced caspase-3 activation compared to controls, suggests that calpain inhibition can be detrimental in doxorubicin-induced cardiotoxicity (Wang, et al. 2013).

To conclude, the activation of apoptotic signalling machinery is tightly linked to ER and mitochondrial stress involved in ischaemic and drug-induced cardiomyopathies, leading to heart failure. The urgent need to establish the contribution of cell death pathways in the various settings that can be used to enable robust high content assays for the development of predictive *in vitro* models of cardiotoxicity is clear. One might argue that the best solution is the multi-parametric acquisition of phenotypes using various cardiotoxicity platforms: the focus of the following sections.

6.3.3.2 High Content Analysis

A large number of *in vitro* models involve the culture of a monolayer of cells adhered to various biological substrates, and hPSC-CM are no exception. Indeed, for a number of assays this is an essential requirement, for example, the MEA recordings and impedance measurements detailed earlier. High Content Analysis (HCA) or High Content Screening (HCS) is widely applied to monolayer cultures in the drug development setting. Using automated microscopy and multi-parameter analysis (usually fluorescent labels or dyes) it allows the quantitative assessment of a range of cellular features. These read-outs include cell death endpoints (e.g. apoptosis, necrosis, autophagy, ER-stress, senescence) and incorporate markers such as

Annexin V/Hoechst (Live/Dead staining), caspase-cleavable antibodies, cleaved PARP-1 and mitochondrial potentiometric dyes. A number of publications, some of which are reviewed next, have described the application of iPSC-CMs in HCA for investigating cardiotoxicity. Patient-derived hiPSC-CMs from donors with a genetic background for monogenic heart diseases (e.g. long QT syndrome, familial hypertrophic cardiomyopathy and familial dilated cardiomyopathy) have also been used in a screening setting to investigate susceptibility to drug toxicity (Liang, et al. 2013). Recent examples of HCA investigated cardiomyocyte proliferation in mouse ESC-CMs (Uosaki, et al. 2013), neonatal rat ventricular cardiomyocytes (Eulalio, et al. 2012) and hiPSC-derived CPCs (Drowley, et al. 2016).

A novel scalable high content microscopy-based assay using hPSC-CMs was developed using a combination of dyes and antibodies that measured nuclear remodelling, mitochondrial status, caspase activation (apoptosis) and loss of cell membrane permeability (necrosis) (Mioulane, et al. 2012). This multi-parametric approach allowed the profile of compounds that acted via caspase-dependent or –independent mechanism of action. Furthermore, exposure of hESC-CMs to hypertrophic stimuli/compounds was assessed by changes in cell size, levels of ANF via antibody labelling and cytoskeletal assembly (Foldes, et al. 2011).

Often, these imaging endpoints are investigated as part of a more 'holistic approach' to both the functional and structural aspects of cardiotoxicity, such as the use HCA following MEA recordings in transparent plates. Following the treatment of hESC-CM with 13 tool compounds for up to 72 h, Clements et al. (2015) demonstrated that the MEA assay can resolve functional but not structural changes, and the HCA assay vice versa (Clements, et al. 2015). The HCA assay involved the assessment of nuclear morphology/DNA content, cell viability, mitochondrial morphology and calcium mobilization. The multi-parameter approach was more informed and flexible, whereby the MEA results could influence whether the HCA was required. For example, if the spontaneous beating in the MEA assay was not perturbed by test compound application it was found that subsequent HCA would be negative for structural toxicity. However, if the compound suppressed spontaneous activity in the MEA assay, the cells could be stained for indicators of cardiomyocyte structure, to determine if/how subcellular structures (e.g. mitochondria) had been impacted. It was suggested that this would provide a more detailed account of a drugs' impact on cardiac physiology than the combined MEA-impedance assay platforms.

Pointon et al. (2013) compared the effects of 34 structural cardiotoxins and 32 non-structural cardiotoxic compounds. Following 6, 24 or 72 h exposure to hESC-CMs and the H9c2 cell line high content imaging was used to assess endoplasmic reticulum integrity, calcium mobilization, membrane potential and mitochondrial membrane potential (Pointon, Abi-Gerges et al. 2013). Following image acquisition ATP content/cell number was also measured. In general, the imaging parameters gave greater sensitivity than ATP depletion (as a whole-well-based assay) for the detection of structural cardiotoxicity at therapeutically relevant concentrations. When the therapeutic concentration (Cmax) of each compound was compared to the IC50/EC50 value generated in each assay the compounds could be classified into mechanistic categories. Changes in either mitochondrial membrane potential or calcium mobilization, in isolation or combination, with other parameters were found to be characteristic of 11 of the 15 structural cardiotoxins evaluated. The beating phenotype of the hESC-CM was also essential for the sensitivity of the assay; when beating was reduced by the co-administration of a negative inotrope (BTS) concentration effect-curves were shifted to the right. Shorter compound exposures (6 and 24 h) did not allow insight into the mechanisms of toxicity due to the lack of detection of structural cardiotoxins at these time points. Overall, it was concluded that hESC-CMs provided a high-throughput experimental model to detect structural cardiotoxicity liabilities in novel compounds at therapeutically relevant concentrations.

Guo et al. (2015) published detailed protocols to demonstrate the presence and function of the ErbB2 signalling pathway in hiPSC-CM and the effect of tyrosine kinase inhibitors (Guo, Eldridge et al. 2015). They developed a multifunctional testing strategy to enable mechanistic studies of investigational anticancer

drugs. The multifunctional approach integrated (1) multi parameter imaging and selected biochemical endpoints as a measure of viability, (2) cardiomyocyte function by impedance and field potential measurements and (3) identification of targets of interest and activation state, and protein loss using siRNA knockdown techniques. Prior to cell use, quality control analysis of the iPSC-CM cells included cell population purity (staining for cTnI (thin filaments) and/or myomesin (functional sarcomere structure)) and the presence and rate of rhythmic contractions. The endpoints assessed were nuclear morphology and cell count, mitochondrial membrane potential, caspase 3/7 activation, ATP content, LDH Assay and cTnT release. These were routinely performed alongside either the xCELLigence RTCA Cardio (impedance and contractility) or CardioECR (impedance, contractility and field potential electrophysiology) platform recordings. Protein expression, both abundance and post-translational modifications, were quantified using automated, Western blot assays. This integrated approach confirmed the presence of functional, integrated signalling pathways and that inhibition of ErbB2 signalling by trastuzumab potentiated the effects of doxorubicin in hiPSC-CMs.

Sirenko et al. (2013) investigated the treatment of hiPSC-CM for 30 minutes or 24 h with 131 drugs (107 positive and 24 negative for *in vivo* cardiotoxicity) across a concentration range in 384-well plates (Sirenko, et al. 2013). Fast kinetic imaging was used to monitor changes in cardiomyocyte function using intracellular Ca^{2+} flux readouts. High content imaging was used in parallel to assess cell viability and mitochondrial integrity and found that beat rate and several peak shape parameters were good predictors of cardiotoxicity, whilst cell viability markers had poor classification accuracy. Grimm et al. (2015) studied the physiological effects of approx. ten compounds on exposure to hiPSC-CM by measuring calcium flux and GPCR activity. In addition, HCA following compound exposure for up to 24 h was used to assess cytotoxicity, mitochondrial integrity and ROS formation (Grimm, Iwata et al. 2015). ROS formation was observed after 30 m treatment, but had returned to basal levels by 24 h. Loss of mitochondrial integrity and cell viability was more apparent following 24 h compound exposure. The tool compounds assessed generated the anticipated results, whilst extracts of commercial gas oils generated biphasic responses, that is increasing beat frequency and loss of beating at low and high concentrations, respectively. Although both studies were limited to 24 h treatment duration, they are good example of a multi-parametric approach for drug profiling.

Although traditionally HCA has been performed in monolayer cultures to allow algorithms to clearly define cell borders, instrumentation developments have resulted in high content confocal imaging, opening up the possibility of similarly assessing complex or 3D culture models (e.g. spheroids: see Section 6.3.3.5.1) (Celli, et al. 2014).

6.3.3.3 Impedance Assays

The impedance technology was introduced earlier in the electrophysiology endpoints and the since the cells are not damaged or sacrificed as a result of these measurements, leads to the opportunity to make multi-parameter assessments on the same cultures. In addition, the impedance the technology should lend itself to investigating the effects of chronic compound exposure, although to date there is little evidence of long-term applications (>72 h). Compound-related toxicity mediated by various effects on action potentials, calcium flux and mechanical contraction will all have consequences that will be captured by impedance assays. Peters et al. (2015) have reviewed the advances in cardiomyocyte impedance with emphasis on hPSC-CM models for toxicity screening and small molecule kinase inhibitors (Peters, et al. 2015). They referred to the impedance as the 'summed response' of cardiomyocyte beating; an integrated, downstream measure of cardiomyocyte function (Peters, et al. 2015). Indeed, caution should be used for arrhythmic effects when the impedance generated 'Cell Index' is also reduced (at 3 or 30 h) since beat pattern disruptions could be caused by cytotoxicity rather than an ion-channel specific effect (Peters, et al. 2015) .

Therefore, from a toxicity perspective the impedance measurements are suited to a broad-based hazard detection screen, but will require additional endpoints for mechanistic understanding not revealed by the impedance measure (Doherty, et al. 2015). Fortunately, the label-free/non-invasive monitoring allows cells to be collected at the end of the study for such further biochemical analysis, as highlighted next. Alternatively, plates where some of the microelectrode sensors have been removed from each well to create an optically clear area would allow for visual inspection of cells during the experiment and/or high content analysis at the end of a study.

Doherty et al. (2015) assessed 24 compounds in hiPSC-CM using a multi-parameter approach. The test system used the xCELLigence platform for beating activity with additional 'structural' endpoints, for example viability, ROS generation, lipid formation and troponin secretion. Following 24 or 48 h treatment, the cardiac beating endpoint (xCELLigence) was the most sensitive at identifying drugs with known clinical effects, however, it did not distinguish between structural or functional effects. Therefore, the additional 'structural' endpoints assessed provided insight into some of the mechanisms of cardiotoxicity. Guo et al. (Guo, et al. 2015) (see High Content Analysis section) also used a multi-parameter approach, including impedance measurements, to investigate integrated ErbB2 signalling pathways and the effect of tyrosine kinase inhibitors.

Disadvantages of the technology are the non-reusable electrode-containing plates and the culture of cells as monolayers to ensure good contact with the culture surface/electrodes. If extracellular matrix (ECM) is required for attachment, it should be a thin layer to allow for the required contact, thereby, potentially limiting its application to more complex and 3D cardiomyocyte models.

6.3.3.4 SeaHorse Flux Analysers

SeaHorse instruments are all designed to interrogate cellular metabolism. Depending on the assay conditions employed, the analysers determine real time, *in vitro* oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as surrogate markers for oxidative phosphorylation, glycolysis or fatty acid oxidation. As such they can be applied to a variety of cell types. The SeaHorse analysers are available in either 24- or 96-well formats, however, due to the design of the plates the growth areas (and hence cell requirements) are less than for the equivalent standard formats. The instrument is temperature controlled and the plate cartridges allow for the automated addition of stock solutions (e.g. compound treatment, substrates, inhibitors) directly into the culture wells followed by mixing and recording. The SeaHorse assay is an endpoint assay, and depending on the assay design the duration is 2–3 h, limiting throughput to a few plates per day. Each cell type investigated benefits from assay optimization (i.e. investigating optimal substrate and inhibitor concentrations) before the effects of compounds are investigated.

Since cardiomyocytes have high metabolic requirements, mitochondrial function/altered cellular energetics may be a useful endpoint with which to investigate drug-induced cardiotoxicity. Early adoption of the SeaHorse technology employed the use of neonatal rat ventricular cardiomyocytes, with studies focussed on elements of mitochondrial function in cardiac hypertrophy (Yu, et al. 2005; Lu and Anderson 2015). However, there have been some reports of the application of this technology to iPSC-CM cultures and cardiotoxicity.

Rana et al. (2012) characterized mitochondrial function in hiPSC-CM when they were maintained in culture media containing different levels and combinations of glucose, galactose (GAL) and fatty acids (FA) (Rana, et al. 2012). Substrate effects were assessed by measuring adenosine triphosphate (ATP) production, the relative contributions of glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) to the bioenergetic profile and gene expression profiles. They found that hiPSC-CMs cultured in GAL-supplemented media resulted in ATP production predominantly through mitochondrial respiration (e.g. higher OCR/ECAR ratios [indicative of energy generation through OXPHOS], greater reserve capacity and higher expression of some mitochondrial complex proteins). Since these conditions most closely mimicked the *in vivo* situation,

they were used to assess six anti-cancer, kinase inhibitor drugs for their effects on oxygen consumption and ATP content at day 21. The rank order of cytotoxicity was sorafenib > sunitinib > dasatinib > imatinib > lapatinib > nioltinib, which appeared to be in agreement with the literature for these kinase inhibitors leading to clinical cardiotoxicity. They concluded that hiPSC-CMs growing in GAL and GAL + FA were more susceptible to mitochondrial toxicants suggesting that such cultures may be more proficient in identifying cardiotoxicants with mitochondrial liabilities.

One of the attractions of hiPSC is the ability to generate patient-derived hiPSC-CMs. Guan et al. (2014) used the SeaHorse technology, alongside a variety of additional assays, to assess the cardiomyocyte phenotype of hiPSC-CMs isolated from normal and Duchenne muscular dystrophy patients. Although there were some differences between the hiPSC-CMs, for example calcium handling and response to injury, no differences were observed in cellular metabolism (i.e. SeaHorse Mito Stress test) between the two phenotypes (Guan, et al. 2014). The SeaHorse assay plates govern that hiPSC-CMs are cultured in monolayers, and therefore, the phenotype is likely to remain immature. Spheroid cultures can be assessed with this technology using adapted plates but no application of hiPSC-CM spheroids and SeaHorse technology was found.

6.3.3.5 Complex and 3D Models

The area of complex, co-culture and 3D cell cultures is undergoing a rapid expansion at present, and it is considered that the culture of cells within a more structurally-relevant environment will lead to improvements in cell phenotype and ultimately improved predictive value. However, despite different results often being obtained between 2D and 3D systems very little validation or evidence of translation to either the *in vivo* or clinical situation has been published. Therefore, it is highly unlikely that one *in vitro* model will be appropriate for all applications and validation in terms of recapitulating the *in vivo* situation is an important consideration.

One assay that has been validated is the embryonic stem cell test (EST, Invitox #113). This ECVAM (European Centre for the Validation of Alternative Methods) test is a validated, *in vitro* assay that can form part of an integrated testing strategy for the identification of embryotoxicants. The compound-related inhibition of differentiation of mouse embryonic stem cells into contracting myocardial cells, when cultured as embryoid bodies, is one of the key endpoints monitored as part of this embryotoxicity assessment (Seiler and Spielmann 2011).

Most 3D culture models for cardiovascular research have been created with a regenerative medicine focus, but more recently are attracting interest from drug screening and disease modelling areas (Zuppinger 2015). The heart consists of highly differentiated cells in an intricate arrangement, with neuronal innervations and a vascular system, which act in concert to respond to the rapidly changing demands of the body. Despite the well documented drawback of hPSC-CMs being their immature phenotype, the longevity of hPSC-CMs in culture and their co-culture with cardiac fibroblasts and endothelial cells are beginning to influence some of the previous limitations of primary cardiomyocyte models (Lundy, et al. 2013). Zuppinger (2015) recently reviewed cardiovascular 3D cell culture from a drug screening perspective, with a focus on engineered heart tissues (EHTs), spheroids, cell sheets and vascular 3D models (Zuppinger 2015).

6.3.3.5.1 Cardiomyocyte Spheroids Spheroids (or microtissues) are small (approximately 200 μm diameter), three dimensional aggregates of cells that undergo self-assembly without the requirement for extracellular matrices. They can be formed from a number of different cell types either using low attachment plates or by hanging drop methods, and are routinely cultured in a 96-well format although 384-well formats are also an option. The spheroid size needs to be tightly controlled to avoid diffusion limits and the formation of a central necrotic core. As with all 3D *in vitro* models, it remains unclear how deep into any structure tool compounds will penetrate and the influence this has on the observed results. Good reproducibility between spheroids/wells is reported leading to low variability, although manual handling and cytotoxicity can result in spheroid loss. Automation of some of these processes may help to resolve some of the

issues described previously and dramatically increase throughput in the drug development setting. HiPSC-CM spheroids have been cultured and take around four–five days to form and start spontaneously beating; they have then been maintained for up to 30 days. Characterization has included morphological assessment of cardiomyocytes and sarcomere formation (e.g. actinin, cardiac troponin T, myomesin, Z-discs) and fibroblasts (e.g. vimentin).

Since cardiomyocytes comprise approximately 30% of the myocardial tissue with non-myocytes, for example fibroblasts and endothelial cells, accounting for the rest, it is thought that these non-myocytes are essential to myocardial structure and function. There is also emerging evidence to suggest they may play important roles within drug-induced cardiovascular toxicity (Cross, et al. 2015), and that co-culture may help to improve the cardiomyocyte maturity (Kim, et al. 2010). Hence, spheroids can be formed of cardiomyocytes alone or in co-culture with cardiac fibroblasts and/or endothelial cells (Ravenscroft et al., 2016). However, it appears that the self-aggregation, spheroid formation of endothelial cells co-cultured with cardiomyocytes does not result in vessel formation. Since the cardiomyocytes and culture plastics are commercially available there is the potential for in house cultures to be established or some CRO organizations are also available to run such studies.

Beauchamp et al. (2015) have reported an extensive variety of characterization studies on hiSPC-CMs cultured as 2D monolayers and 3D spheroids (Beauchamp, et al. 2015). For example, the cardiotoxic kinase inhibitor, sunitinib, caused cell necrosis following overnight treatment as demonstrated with Live/Dead staining. Cardiomyocyte spheroids treated with phenylephrine demonstrated an increased beat frequency maintained over 30 days in culture. Spontaneous calcium transients were measured by the Ca^{2+} sensitive probe, fluo-4, however it was noted that it only penetrated the first outer layers of the spheroid. The spheroids also responded to electrical field stimulation and caffeine (ryanodine receptor agonist stimulating Ca^{2+} release from the sarcoplasmic reticulum). Using IonOptix (a video motion tracking system) they assessed contractile activity: isoproterenol increased beat frequency; blebbistatin decreased the amplitude of the contractions until they ultimately stopped; Doxorubicin changed the regularity of the contractions. They concluded that cardiomyocyte spheroids were worthy of further study as a new and promising relevant model for the evaluation and development of new therapies and detection of cardiotoxicity.

Using confocal, high content analysis spheroids can be investigated using similar endpoints to those described previously for monolayer cultures, for example Hoechst DNA staining, calcium homeostasis using Fluo-4 AM, and mitochondrial function using TMRE. Also in line with monolayer cultures, cellular ATP content can be measured once imaging is completed. Using nine known cardiotoxins and two negative controls, Ravenscroft et al. (2015) reported that the tri-cultured cardiac spheroids had a better positive compound identification compared to 2D monolayer cultures (Ravenscroft, et al. 2015). In addition, the cardiomyocyte only spheroids have also been used in the assessment of hypertrophy, following an extended treatment over 14 days and bright field imaging to monitor spheroid size. At the end of the treatment period cytotoxicity was also investigated by propidium iodide staining. Using microtissue area as the endpoint, cardiomyocyte hypertrophy (prior to gross cytotoxicity) was correctly predicted for all tool compounds (seven studied) except isoproterenol, although it was unclear why this tool compound should be ineffective.

Cardiomyocyte spheroids have also been assessed using voltage sensitive dyes and shown to have a shorter action potential duration than in 2D culture (Rodriguez 2015). Differences were also observed in the sensitivity of well characterized ion channel antagonists between monolayer and spheroid cultures. However, the lack of a consistent pattern in sensitivity demonstrates the requirement for appropriate validation of emerging assay platforms.

A perceived advantage of spheroids is the ability to self-aggregate without the requirement of a scaffold consisting of artificial materials or ECM proteins. However, it should be noted that scaffold-based constructs are being investigated to promote the linear alignment of cardiomyocytes in an attempt to positively influence the maturation phenotype.

Another expanding area of complex and 3D *in vitro* models is the incorporation of microfluidics. Data on the incorporation of cardiomyocyte spheroids/embryoid bodies into such platforms is emerging (Bergstrom, et al. 2015; Rismani Yazdi, et al. 2015), but at too early a stage to determine any potential benefits. Data is also awaited from the Innovate UK funded 'CVTox model' (Development of a Co-Culture Cardiovascular Toxicological Model by Kirkstall). This project is focused on developing a co-culture of human cardiomyocytes, smooth muscle cells and endothelial cells and incorporating re-circulating flow.

6.4 Conclusions and Future Direction

Increasingly the pharmaceutical industry is seeking new strategies to make improvements in late-stage attrition reduction, both in terms of efficacy and safety. Advances in *in vitro* cellular models provide access to what is hoped will be more physiologically and human-relevant platforms. This review has demonstrated how the availability of large numbers of consistently produced stem cells, differentiated into cardiomyocytes has significantly impacted the area of cardiotoxicity assessment in recent years, although comprehensive validation is still required for many platforms. The most recent advancements in isolating and differentiating hPSC-CMs from patient populations have also increased the potential for studying drug-related effects in disease-relevant systems. Continued focus in the area of enhancing the maturity of the cardiomyocyte phenotype along with novel experimental designs using complex and/or 3D cell culture, and technologies, will only serve to expand the field in the future. Whilst this review has focussed on those platforms that can either currently be employed or will be applicable in the very near future, the area of more complex systems such as 'organs-on-a-chip' and organoids will doubtless continue to draw attention as they develop.

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7

Disease-Specific Stem Cell Models for Toxicological Screenings and Drug Development

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7.1 Evidence for Stem Cell-Based Drug Development and Toxicological Screenings in Psychiatric Diseases, Cardiovascular Diseases and Diabetes

7.1.1 Introduction into Stem-Cell Based Drug Development and Toxicological Screenings

Drug development aims for the discovery of safe and effective drugs for the treatment of human diseases. Many different methodical approaches are intertwined during drug development including computational design, *in vitro* cell culture applications, *in vivo* animal models, and the subsequent execution of clinical trials. For example, computational methods play a major role in the discovery of drugs and computational drug development is a fast growing scientific field (Sliwoski et al., 2014). *In vivo* animal models are a tool to verify safety and effectivity in a living organism before tests in humans can be performed. A variety of different models have been established in many species (Nestler and Hyman, 2010). However, *in vitro* cell models are the method of choice to evaluate large substance groups and the development of disease-specific stem cell models has substantially broadened the field of application. They are suitable for large-scale production and high throughput screens (HTS) and, compared to animal models, require fewer human and technical resources and time. In the past, cell models have been thought to be less meaningful for translating scientific results to the human system than *in vivo* models applying a living organism (Begley and Ellis, 2012).

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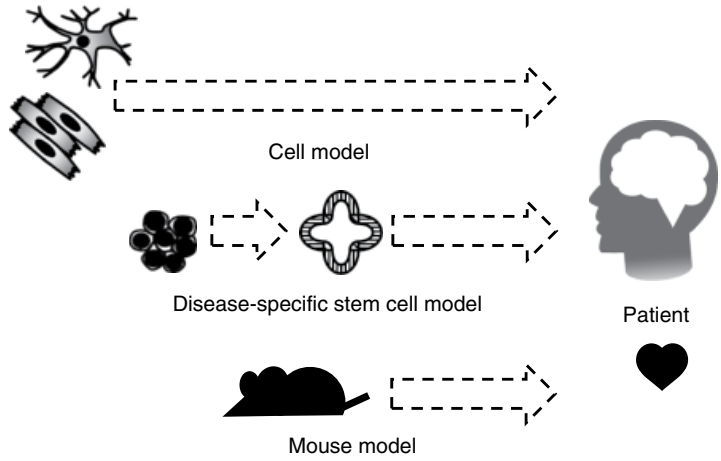

Figure 7.1 *Disease-specific stem cell models represent a link between cell models and animal models in drug development and toxicological screenings*

This remains to be true for basic cell models, but the development of disease-specific stem cell models provides a link between basic cell models and studies applying animal models (Figure 7.1).

Drug development usually starts with a target-based or a phenotype-based approach for the analysis of large chemical libraries in engineered cell lines by HTS. Target-based drug development (also termed reverse pharmacology) usually relies on specific molecules that are affected in a certain disease. HTS aim at identifying those substances that inhibit, modulate or activate the target. It is well accepted that drug development based on molecular targets has advantages over phenotype-based approaches (Zheng et al., 2013). However, there are limitations. For example, the success of target-based screens used for drug discovery was described to be lower for the development of small molecule drugs in comparison to phenotype-based approaches (Swinney and Anthony, 2011). In phenotype-based drug development (also termed forward pharmacology), the molecular mechanism and the target remain unknown even after the drug's efficacy is determined. The aim of phenotype-based studies is to rescue cells with a disease-specific phenotype, which therefore may contribute to a higher success of this kind of screenings.

Toxicological screenings are applied for the analysis of toxins and poisons. Even drugs may represent a toxin when improper concentrations are applied. Toxicological screenings during drug development are necessary to estimate the effects, side-effects, and other adverse reactions in response to the application of a certain drug in humans such as analyzing the toxicological effects of lithium in zebrafish (Siebel et al., 2014).

The technological advances in the field of stem cell research and the application of stem cells in 3D disease-specific models strongly improved the applicability of cell models and strongly reduced the gap to human *in vivo* conditions. We will review the recent findings in the field of disease-specific stem cell models for psychiatric and cardiovascular diseases.

7.1.2 Relevance for Psychiatric and Cardiovascular Diseases

Psychiatric diseases such as Alzheimer's disease (AD) and cardiovascular diseases (CVD) are among the major causes of suffering and death in the United States of America (USA), in Europe, and in other industrialized countries. The number of patients is growing in correlation to the number of elderly people in our societies (Alzheimer's Association, 2015; Tuomi et al., 2014; Yusuf et al., 2015). Especially the risk for AD and CVD is strongly elevated with the age of patient. Accordingly, there is a growing interest in the ageing society.

AD and CVD are multifactorial diseases. Research in the field of human genetics revealed that single nucleotide polymorphisms (SNPs), copy number variations (CNVs) and even functional homozygous mutations confer a risk for these diseases and influence their onset, progression, and/or their treatment (Stankiewicz and Lupski, 2010). CNVs act through a variety of molecular mechanisms, but most often a dosage effect of the affected genes inside the CNV is described. For example, the Potocki–Lupski syndrome results from a haploinsufficiency on chromosome 17p11.2 leading to a neurobehavioral disorder also including cardiovascular abnormalities (Slager et al., 2003). However, there are microdeletions, which have been identified in autism, epilepsy, and SCZ as well (Sharp et al., 2006). There are a lot of association studies focusing on the analysis of risk factors for psychiatric diseases and CVD. Such risk factors or risk loci provide a target for target-based drug development. Even the analysis of risk loci with an unknown function provides a target for drug development by the application phenotype-based approaches.

The United States National Institute of Health (U.S. NIH) provides a database (clinicaltrials.gov, accessed February 26, 2016), which currently lists many clinical trials (US and non-US) analyzing a variety of chemical compounds for the treatment of AD (1746 trials) and CVD (3752 trials). There are promising results in pre-clinical studies and early clinical trials for therapeutic drugs or antibodies, but most of the studies have been withdrawn or failed to work in patients (De Felice and Munoz, 2016). The high number of failed clinical trials highlights the fact that drug development is predominantly affected by the difficult translation of animal models into the human system.

New promising approaches for drug development arise from the analysis of the disease- and patient-specific genetic background. The complex and multigenic mechanisms, which confer a risk for a certain disease are poorly understood, but DNA variations as well as mutations are suggested to provide potential targets for drug development. Genome-wide association studies (GWAS) for AD (Lill et al., 2015), CVD (Padmanabhan et al., 2010), and type 2 diabetes melltius (T2DM) (Shungin et al., 2015) have recently been described by a variety of genetic loci with growing European and American cohorts. The technological advance in the field of RNA and DNA sequencing will further accelerate and enlarge the execution of such studies. Additionally, meta-analysis projects provide a strongly growing scientific field for AD (Lambert et al., 2013), CVD (Tragante et al., 2014), and T2DM (Dastani et al., 2012).

Epidemiological and clinical studies suggested comorbidity between psychiatric diseases such as schizophrenia (SCZ) or AD, CVD, and diabetes. For example, risk loci were confirmed to predispose for SCZ and T2DM as well (Alkelai et al., 2012). Another study demonstrated that the detection of common SCZ variants is improved when the pleiotropy of CVD risk loci is taken into consideration (Andreassen et al., 2013). The authors further suggest a mechanistic relationship between SCZ and CVD risk factors. Association studies revealed that psychiatric diseases, CVD, and diabetes can lower or increase the comorbidity. GWAS performed within a Czech population suggested that AD patients have a lower risk for stroke, hypertension, and diabetes (Sery et al., 2014). Analysis of another cohort found that cardiovascular diseases contribute to AD (Liu et al., 2014a). The different observations might contribute to an age-dependent correlation of hypertension and cognitive impairment (Qiu et al., 2005).

The scientific field of association studies recently revealed a variety of potential targets for drug development. These potential targets need to be verified and further analyzed by *in vitro* and *in vivo* models (Figure 7.1).

7.1.3 Advantages of Human Disease-Specific Stem Cell Models

Rodents are broadly and increasingly applied for disease modeling, but notably these animals poorly mimic complex human diseases. Even though at a first glance organ development in rodents and humans is roughly comparable there are still significant differences in molecular and cellular mechanisms. Accordingly, the analysis

of disease mechanisms is limited by these species-specific differences. Several studies raised evidence that human *in vitro* models are more suitable than animal models for complex human diseases including neurodevelopmental diseases such as microcephaly (Lancaster et al., 2013), diabetes (Jennings et al., 2013), and inflammatory diseases (Seok et al., 2013). One can conclude that species-specific differences represent a major road block for drug development.

The same holds true for toxicological testing. Many tests in rodents or other animals did not faithfully represent the human response. For example, thalidomide showed dramatic teratogenic effects in humans and over 10,000 children were born with malformations (Vargesson, 2015). Thalidomide is still used successfully for treatment of multiple myeloma and other diseases in non-pregnant patients.

In vitro models provide a robust system for the analysis of targets in response to the treatment with a certain drug. The analysis of cellular and intracellular molecular signaling pathways is much easier when *in vitro* models are applied. For example, mood stabilizers alter a variety of intracellular signaling pathways including the phosphoinositol cycle, Wingless-realted integration site (WNT) signaling, and signal transduction by neurotrophic factors (Coyle and Duman, 2003). Cell lines regularly grow as monolayers enabling the analysis of single cells and cell populations as well. However, the majority of cell lines lost their natural characteristics due to their immortalization. Accordingly, the analysis of mature and functional properties in cell lines is often restricted to a certain aspect or basic cellular signaling pathways. For example, glioblastoma cell lines are derived from brain tumors, which artificially retained a subset of mature or functional properties of certain glia cells (Dunn et al., 2012).

More advanced disease-specific cell models combine basic cell models with disease-specific aspects (Figure 7.1). Disease-specific aspects may be reflected by mimicking the (1) differentiation and maturation status of certain cells, (2) the specific cell composition of a given tissue, (3) physical impulses necessary for maturation, and (4) the 3D structure of the tissue. Organoids can be obtained from pluripotent and adult stem cells representing a powerful tool to mimic all the issued aspects. Adult stem cells can be isolated from stomach, small intestine, and colon by the isolation of LGR5-positive adult stem cells in glands and crypts (Ren et al., 2014; Sato and Clevers, 2015). Pancreas and liver organoids can be established from duct cells also representing a source for adult stem cells (Huch et al., 2013; 2015). Patient-derived pluripotent stem cells offer the opportunity to study the genetic background of the patient, which can potentially represent a major disease-specific aspect.

7.1.4 Pluripotent Stem Cell Models

During human development, cells from the inner cell mass (ICM) can be explanted from the blastocyst and propagated as permanent cell lines, which are well described as embryonic stem (ES) cells (Wobus and Boheler, 2005). ES cells are pluripotent, which is defined by virtually unlimited proliferation and the capability to differentiate into every cell type of the human body. Accordingly, the analysis of pluripotency is a prerequisite for the analysis of toxins and poisons in stem cell-based toxicological screenings for embryotoxicity. Even though genetically engineered mouse models were shown to be suitable for mimicking certain aspects of the human embryonic development for drug testing and toxicological screenings (Wobus and Loser, 2011), the establishment of human ES cells in 1998 (Thomson et al., 1998) increasingly replaced murine stem cell models.

Accordingly, the discovery of human ES cells also allowed the generation of adequate cell material for cell replacement therapies. In 2010, the Food and Drug Administration (FDA) of the USA approved two clinical trials, which applied human ES cell derivatives for replacement therapies. Oligodendrocyte progenitors were used to treat spinal cord injury (ClinicalTrials.gov identifier: NCT01217008) and retinal pigment epithelial cells were applied to treat dry age-related macular degeneration (ClinicalTrials.gov identifier: NCT01344993) (Grabel, 2012; Schwartz et al., 2012). The trial analyzing macular degeneration was successful leading to

more clinical trials analyzing human stem cell-derived transplants as a cure for macular dystrophy and macular degeneration (Kimbrel and Lanza, 2015). The trial analyzing spinal cord injury was discontinued by the Geron Corporation in 2011. Asterias Biotherapeutics acquired the assets of the clinical trial in 2013 and received an approval from the FDA in 2014 (Kimbrel and Lanza, 2015) to conduct a new clinical trial. Another study, initiated in 2013, uses human ES cell-derived cardiac progenitors for the transplantation into patients suffering from severe heart failure (ClinicalTrials.gov Identifier: NCT02057900).

The generation of ES cells requires the destruction of fertilized eggs and therefore is ethically disputed (Lo and Parham, 2009). Reprogramming of somatic cells is thought to provide an equivalent cell type without ethical concerns.

7.1.5 Reprogramming of Somatic Cells for Disease-Specific Stem Cell Models

In 2006, the Shinya Yamanaka group generated a completely new source of pluripotent stem cells, the so-called induced pluripotent stem cells (iPS cells). In 2012, the Nobel Committee awarded Shinya Yamanaka and Sir John Gurdon the Nobel Prize in Physiology or Medicine "for the discovery that mature cells can be reprogrammed to become pluripotent". This technique also allows generating disease-specific iPS cells. Such disease-specific *in vitro* models are useful for cell replacement strategies, drug development and toxicological screenings. In contrast to human ES cells, human iPS cells do not require the destruction of human blastocysts. The forced expression of POU class 5 homeobox 1 (POU5F1; also known as OCT4), SOX2, Kruppel-like factor 4 (KLF4), and v-myc myelocytomatosis viral oncogene homolog (C-MYC) (OSKM, Yamanaka factors) is sufficient to reprogram mouse fibroblasts into ES cell-like cells (Takahashi and Yamanaka, 2006). Human iPS cells were first generated in 2007 by the same group using an identical set of transcription factors (Takahashi et al., 2007). In parallel, the group of James Thomson successfully applied OCT4, SOX2, nanog homeobox (NANOG) and cell lineage abnormal protein 28 homolog to *Caenorhabditis elegans* A (LIN28) (ONSL, Thomson factors) for the production of human iPS cells (Yu et al., 2007). Human iPS cells and human ES cells share almost all characteristics of pluripotent cells. However, gene expression profiles of different iPS cell lines are very similar, but not identical to human ES cell lines (Polouliakh, 2013). Differently expressed genes are retained from the somatic donor cell or induced during reprogramming. Reprogrammed cells also require human ES cell-specific culture conditions including co-culture with mouse embryonic fibroblasts (MEFs) or culture on Matrigel™ and other substrates. Many laboratories have successfully generated iPS cells from different cell types using different innovative methods (Mostoslavsky, 2012; Robinton and Daley, 2012). The choice of different cell types combined with the diversity of reprogramming procedures leads to different reprogramming efficiencies and varying iPS cell qualities.

7.1.6 Transdifferentation of Somatic Cells for Disease-Specific Stem Cell Models

The generation of human iPS cells aims at the generation of permanent pluripotent cell lines most similar to human ES cells. Subsequently, iPS cells are differentiated to generate a certain cell type or cell population. Transdifferentiation protocols aim at the generation of certain cell types or cell populations without passing through a stable pluripotent stage. One can methodically divide transdifferentiation procedures into two groups, namely (1) direct reprogramming and (2) direct conversion (Prasad et al., 2016). During direct reprogramming cells pass through the pluripotency stage, but during direct conversion cells bypass the pluripotent stage. Direct reprogramming protocols are methodically very similar to reprogramming protocols, but they are much less time consuming by skipping a permanent and stable pluripotent or multipotent stage (Prasad et al., 2016).

Viral transfection is the most efficient method for the generation of iPS cells, but there are many safety concerns about the applicability of viral generated iPS cells (Okita et al., 2007). Therefore, many non-viral and non-integrating methods have been established and have been proven to be adequate techniques (Brouwer et al., 2016).

Epigenetic modification of DNA and histones occurs during embryonic development (Vastenhouw and Schier, 2012; Zlotorynski, 2016). During reprogramming, the epigenetic information of the genome is almost erased. However, several studies reported that reprogrammed cells retain a somatic/epigenetic memory (Lister et al., 2011). This epigenetic memory is an important issue for the analysis of disease-related epigenetic modifications. For example, it has been demonstrated that disturbed DNA methylation of the brain-derived neurotrophic factor (BDNF) contributes to psychiatric disorders (Ikegame et al., 2013). Transdifferentiation protocols may provide a useful *in vitro* model for the analysis of patient-specific epigenetic modifications.

Direct conversion protocols benefit from somatic memory when a related donor cell type is applied. Direct conversion of mesodermal cells for the generation of cardiomyocytes was first demonstrated by the forced expression of myocard master genes in mice (Takeuchi and Bruneau, 2009). Likewise, the conversion of exocrine pancreatic cells into endocrine pancreatic cells (Klein et al., 2015) and the conversion of astroglia into neurons via forced expression of the master regulator paired box 6 (PAX6) (Hack et al., 2005) was demonstrated. SOX2 is another neural master regulator, especially during neural induction. Accordingly, using SOX2 human fibroblasts could be transdifferentiated into neural stem cell-like cells (Ring et al., 2012). The generation of functional neurons from fibroblasts was shown by the forced expression of Achaete-scute family bHLH transcription factor 1 (ASCL1), Brain 2 (BRN2, officially termed POU3F2), and Myelin transcription factor 1 like (MYTL1) (Vierbuchen et al., 2010) in murine cells and by adding NEUROD1 in the human cells (Pang et al., 2011).

7.2 Disease-Specific Stem Cell Models for Drug Development in Psychiatric Disorders

The field of disease-specific stem cell models recently revealed various approaches focusing on different tissue-related aspects of diseases. Tissue engineered nerve grafts have been described for peripheral nerve regeneration (Gu et al., 2014). Modeling the blood brain barrier (BBB) was described as a model for studying AD (Lippmann et al., 2012), but BBB models offer the opportunity to study many other diseases affecting proper BBB functionality such as epilepsy, and multiple sclerosis (Naik and Cucullo, 2012). There is also the promising opportunity to study pharmacological aspects of drugs in general (Wong et al., 2013). Brain models for neurodevelopmental psychiatric disorders (Brennand et al., 2012) and traumatic brain injury (Morrison et al., 2011) have been established with the focus on developmental patterning of cells and cell populations within a 3D tissue-specific model. In this regard, microfluidic systems allow testing various chemical and physical stimuli via HTS (Harink et al., 2013).

7.2.1 Disease-Specific Stem Cell Models Mimicking Neurodegenerative Disorder

Neurodegeneration describes the progressive loss of neurons and glia cells or the loss of their functionality in many psychiatric diseases including amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, and AD. Human iPS cell-derived neurons were used for an anti-beta amyloid (Aβ) drug screening for the treatment of AD (Yahata et al., 2011). Human neural precursors were applied for the generation of 3D cultures in Matrigel-based thick-layer 3D cultures (Choi et al., 2014). Rat adrenal gland PC12 cells are an established model for neurotoxicological screenings (Shafer and Atchison, 1991) recently applied in HTS for anti-AD drugs (Hou et al., 2014). SH-SY5Y neuroblastoma cells provide mature neuron-like properties such as

dopamine-β-hydroxylase characteristic of catecholaminergic neurons (Kovalevich and Langford, 2013). Therefore, these cells were recently used as a screening tool for AD drug candidates (Loffler et al., 2012). SH-SY5Y cells were also applied in a HTS assay for cellular levels of the tau protein, which is implicated in the pathology of many neurodevelopmental disorders (Dehdashti et al., 2013). All these models are based on (immature) proliferating cells. However, recently the use of non-dividing fully differentiated mature murine brain cells was reported (Ahn et al., 2016). Ahn and co-authors thus described an *in vitro* culture system for the generation of brain aggregates suitable for modeling neurodegenerative diseases.

BBB models are very important for drug development because the BBB represents the most important interface between blood circulation and the central nervous system (CNS). It maintains homeostasis in the CNS and protects the CNS from bacteria, viruses, and other substances (Obermeier et al., 2013). Accordingly, the BBB also represents a barrier for drugs. It consists of endothelial cells and neighboring cells including pericytes, neurons, glia cells, and microglia regulating the physical and metabolic properties of the barrier. The barrier comprises a network of influx and efflux proteins, but also includes endocytotic activity. The most important transport proteins are the solute carrier (SLC) or the ATP-binding cassette (ABC) transporter family that actively efflux compounds back into the blood circulation including pharmaceutical drugs (Obermeier et al., 2013).

7.2.2 Disease-Specific Stem Cell Models Mimicking AD

AD is the most common type of dementia characterized by a progressive neurodegeneration, brain atrophy, and cognitive impairment (Burns and Iliffe, 2009). There is an increased production and accumulation of Aβ peptides in the brain. These peptides are generated by the sequential cleavage of the amyloid precursor protein (APP). Accumulation of Aβ peptides can result both from the increased production as well as from the decreased deposition (Sagare et al., 2013). Aβ appears to be directly involved in the degeneration of cerebral capillaries that constitute the BBB. Accordingly, BBB models provide a disease-specific model for the analysis of Aβ accumulation. Various transporter systems are involved in Aβ transport including the low-density lipoprotein receptor-related protein (LRP), the receptor for advanced glycation end products (RAGE), the megalin receptor, and the ABC transporter ABCB1 (Obermeier et al., 2013). Aβ enhances microglia and macrophage activation and induces secretion of proinflammatory cytokines and chemokines.

Molecular genetics of AD are complex and show heritability of up to 79% (Hollingworth et al., 2011). AD-specific neurons and glia cells have been generated from AD patients aiming at the analysis of AD phenotypes *in vitro*. However, the studies focused on single gene mutations in APP, PSEN1, and PSEN2 causative for AD in Down syndrome or early onset AD (EOAD) representing about 1% of the AD patients. These mutations usually increase the production of Aβ, but no drug target based on the amyloid cascade has been established. Aβ accumulation is due to the dysregulation of the BBB. The majority of AD patients suffer from late onset AD (LOAD), but only one study focused on LOAD by analyzing a patient heterozygote for the ApoE4. APOE structure and function depends on three major classes of polymorphisms: ApoE2 (cys112, cys158), ApoE3 (cys112, arg158), and ApoE4 (arg112, arg158) (Liu et al., 2013). APOE is poorly understood, but patients carrying the ApoE4 (1) have an elevated risk for LOAD, (2) show earlier onset of LOAD, and (3) suffer from a stronger progression of LOAD (Hibar et al., 2015). The latest meta-analysis of common variants demonstrated that 19 loci, in addition to the APOE locus, harbor variants showing genome-wide significant association with AD (Lambert et al., 2013). More recently, rare variants in APP, ABCA7, and TREM2 were discovered to affect susceptibility to the sporadic, late-onset form of AD (Steinberg et al., 2015). The highly complex structure of the human brain is strongly shaped by genetic influences. Common genetic variants influence the subcortical brain structures highlighting the impact on the brain anatomy and physiology by molecular genetics of AD and other neurodegenerative and neurodevelopmental diseases (Hibar et al., 2015). There is a need to analyze these recently discovered genes and loci in disease-specific

stem cell models. The analysis might contribute to the characterization of known and unknown disease mechanisms providing targets for drug development.

In general, *in vitro* models of the BBB are very strong tools and play a very important role in elucidation of physiological and pathophysiological molecular mechanisms (Bicker et al., 2014). BBB *in vitro* models are indispensable in drug discovery and development to discriminate between CNS penetrant and non-penetrant compounds. An important component of BBB *in vitro* models are brain-derived endothelium cells (ECs), but other barrier forming cells such as the Caco-2 cells are applied (Georgievska et al., 2015). BBB models apply brain-derived ECs for drug screening purposes or more complex cultures with astrocytes and pericytes for physiological and pathophysiological studies. The development of functional BBB *in vitro* models depends on the tightness of cell monolayers for the establishment of the physical barrier. The barrier function can be measured by the transendothelial electrical resistance (TEER) or by the permeability of fluorescent labelled macromolecules. Physiological relevant TEER values of 1000–2000 $\Omega*cm^2$ are only achievable with primary brain-derived ECs from pigs or rats. Immortalized cell lines such as the human cell line hCMEC/D3 has a barrier with TEER values below 150 $\Omega*cm^2$. However, it is necessary to replace basic cell models with more suitable human disease-specific stem cell models. The generation of functional ECs, neurons, and other cells of the neurovascular niche from iPS cells has been described (Lippmann et al., 2012). The application of iPS cell-derived derivatives aims at the development of more reliable and more standardized HTS for drug discovery and toxicity testing.

There are promising reports based on iPS cell derivation and subsequent differentiation into neurons from both familial and sporadic AD (Sterneckert et al., 2014). Sporadic AD-specific iPS models showed oligomerization and increased secretion of Aβ peptides and identified involved signaling pathways including microtubule associated protein tau (MAPT) and glycogen synthase kinase 3 beta (GSK3β) (Hossini et al., 2015; Israel et al., 2012; Kondo et al., 2013). Neurons derived from patients with dominant and recessive APP mutations showed different amyloid-related phenotypes with increased or decreased Aβ peptide secretion in different studies (Israel et al., 2012; Kondo et al., 2013; Mertens et al., 2013). Dominant mutations were also shown to alter MAPT and GSK3β. PSEN1 as well as PSEN2 models revealed an increased Aβ42/Aβ40 ratio (Mertens et al., 2013; Yagi et al., 2011). Accordingly, Trisomy 21 also showed amyloid-related phenotypes (Shi et al., 2012). Drug screenings based on iPS cell technology have been executed for the analysis of candidate drugs with promising results for example for the application of γ-secretase modulators (Liu et al., 2014b). The recently established disease-specific AD models focused on the tau pathology and the amyloid-related phenotypes of EOAD. Disease models relevant for the majority of AD patients suffering from LOAD will require more suitable *in vitro* models based on iPS cells of LOAD patients.

7.2.3 Disease-Specific Stem Cell Models Mimicking Neurodevelopmental Disorders

Neurodevelopmental disorders are caused by abnormalities during the stage of the rapid embryonic development of the CNS leading to an altered structure and function of neuronal and synaptic populations (Gaspard and Vanderhaeghen, 2011). Neurodevelopmental disorders manifest during fetal or early postnatal life until early adulthood and are associated with a life-long suffering. The variety of signs and symptoms includes cognitive impairment (learning disability, mental retardation), abnormal behaviors, sensory and neuromotor dysfunctions (problems with speech and language) as well as seizures. Up to 80% of children with neurodevelopmental disorders are also reported to have disrupted sleep (Geschwind, 2009).

The dopamine and the glutamate neurotransmission system as well as functional changes in astrocytes and microglia have often been implicated to play a role in neurodevelopmental pathogenesis, influencing synaptic development, and synaptic transmission via the uptake of neurotransmitters (Eroglu and Barres, 2010; Molofsky et al., 2012; Schummers et al., 2008). Calcium-mediated alterations in synaptic function and plasticity have also been reported (Haydon and Nedergaard, 2015). Numerous factors acting during early development may

contribute to such alterations, including insufficient maternal nutrition, smoking, infection with various pathogens, psychological stress as well as perinatal injury (Garay and McAllister, 2010; Patterson, 2009; Shi et al., 2003). Likewise, radiation- and chemotherapies in childhood cancers contribute significantly to neurodevelopmental disorders. It has been shown that maternal immune activation leads to region specific changes in brain cytokines and neuropathological changes (Garay et al., 2013; Weir et al., 2015). Even obstetrical complications increase the vulnerability to neurodevelopmental disorders (Elitt and Rosenberg, 2014). Additionally, environmental influences and the patient-specific genetic background were shown to be involved in the manifestation of neurodevelopmental disorders. An example is stressful life events such as childhood maltreatment of children with a functional polymorphism in the promoter region of the serotonin transporter gene (Caspi et al., 2003). Individuals with the short allele have been found to respond more vulnerable to develop depressive symptoms than individuals with the long allele. Another example is the polymorphism in the catechol-O-methyltransferase (COMT) gene. Cannabis use by individuals carrying the valine allele have a higher chance to exhibit psychotic symptoms and to develop neurodevelopmental disorders in comparison to individuals with two methionine alleles (Caspi et al., 2005). Currently, there is no clear explanation why disease-associated genetic variations manifested neurodevelopmental diseases at different ages.

Neural signaling pathways involve hundreds of genes. Severe mutations in any one of these genes may lead to a psychopathological phenotype. There are four subgroups, based on their genetic etiology, namely (1) aneuploidy, (2) micro-deletions, (3) single affected genes, and (4) complex etiology, whereas the latter is thought to be caused by a combination of genetic, environmental and epigenetic factors (van Loo and Martens, 2007). Epigenetic alterations include DNA methylation and posttranslational modifications of histone proteins. Such alterations are responsible for single gene defects also described in the Fragile X syndrome (Jin and Warren, 2000). Disturbed DNA methylation has also been described as a cause for spina bifida (Pitkin, 2007). Epimutations are thought to occur upon exposure to environmental risk factors, including early developmental stress (Rideout et al., 2001). Among others, neurodevelopmental disorders embrace autistic disorders, SCZ, attention deficit hyperactivity disorder (ADHD), bipolar disorder, mental retardation syndrome, Tourette's syndrome, and William's syndrome.

Autistic disorders are a group of complex neurodevelopmental disorders arising in early childhood, affecting 1% of the world's population. Autistic disorders are characterized by qualitative communication impairment, atypical social interaction, and restricted and repetitive patterns of behavior (Acab and Muotri, 2015). There are two mayor forms of autistic disorders, syndromic and nonsyndromic autism. Syndromic autism comprises Fragile X syndrome, Rett syndrome, Timothy syndrome, Angelman syndrome, and Prader–Willi syndrome. Each of them is defined by a set of associated phenotypes where the genetic cause is known and the gene mutation is identified.

Fragile X syndrome is the most common form of syndromic autism, where approximately 70% of the affected children are boys. The disorder is caused by loss of expression of the Fragile X mental retardation 1 (FMR1) located on the X-chromosome (Sheridan et al., 2011; Urbach et al., 2010). FMR1 is associated with synaptogenesis and the Fragile X mental retardation protein (FMRP) can be detected at synapses and dendritic spines (Bassell and Warren, 2008). Fragile X syndrome patients display many impairments including learning disabilities, physical and behavioral, atypical social development, memory problems, and difficulty with face encoding (Kumari et al., 2015). FMR1-knockout mice showed increased density in dendritic spines and altered spine morphology (Penagarikano et al., 2007) and enhanced signaling through the metabotropic glutamate receptor (Huber et al., 2002). Disease-specific iPS cells obtained from Fragile X syndrome patients revealed that CGG trinucleotide repeats influence the CpG methylation of the FMR1 promoter and the FMRP expression (Sheridan et al., 2011). Neurons generated by the differentiation of iPS cells from Fragile X syndrome patients revealed lower synaptic protein levels and fewer synapses, reduced neurite length and abnormal functionality, as well as increased calcium levels (Doers et al., 2014; Liu et al., 2012). The re-establishment of the FMR1 expression was reported in mice by the gamma-aminobutyric acid (GABA) B receptor agonist Arbaclofen with promising results in a preclinical study of Fragile X syndrome (Erickson et al., 2014). A

recent drug screening study reported 6 compounds to increase FMR1 gene expression in neural stem cells differentiated from Fragile X syndrome iPS cells, but none of these compounds resulted in clinically relevant levels of FMR1 (Kumari et al., 2015).

Besides the Fragile X syndrome model, only a few stem cell-based models have been established. Rett syndrome is another syndromic autism spectrum disorder caused by mutations in the CpG binding protein (MECP2). The generation of mouse iPS cell-derived neurons from MECP2-deficient mice revealed a disturbed sodium channel function (Farra et al., 2012). However, there is a need to establish human disease-specific *in vitro* models.

Nonsyndromic autism is a prevalent disorder with complex changes over time from early childhood into adulthood. Among many other serious symptoms, patients suffer from impaired language development, stereotyped behavior, epilepsy, hyperactivity, and aggression (Geschwind, 2009). The current diagnosis of nonsyndromic autism is based on behavioral observation and developmental history and shows a heritability of up to 50% (De Rubeis and Buxbaum, 2015). Recently, risk loci have been identified, but the found risk variants were rare and no causative gene or signaling pathway has emerged (Jeste and Geschwind, 2014). However, the affected loci carry genes involved in synaptic transmission, protein synthesis, and epigenetic regulation (Beg and Geschwind, 2012) and a subset of the found risk loci was recently described in other neurodevelopmental disorders such as SCZ. Amongst others, the list of risk genes for nonsyndromic autism includes FMR1, GABRB3, reelin (RELN), N-methyl-D-aspartate receptor (GRIN2B), contactin-associated protein-like 2 (CNTNAP2), engrailed homeobox 2 (EN2), neuroligins (NLGNs), neurexins (NRXNs), and the oxytocin receptor (OXTR) (Penagarikano et al., 2007; Selby et al., 2007; Sgado et al., 2013). These candidate genes hold great potential for the analysis of autism spectrum disorders. They need to be further analyzed including disease-specific stem cell models.

7.2.4 Disease-Specific Stem Cell Models Mimicking SCZ

SCZ is a severe and devastating mental disorder affecting 1.1% of the world's population (Brennand et al., 2012). It is caused by a combination of genetic, biological, psychological, and environmental factors (Akbarian, 2014). The disease begins in prenatal or perinatal life, but generally symptoms emerge in early adulthood, in most male patients between 15 and 24 and females between 20 and 29, and 45 and 50 years of age. SCZ can be divided into four stages, from risk to prodrome to psychosis to chronic disability (McGorry et al., 2008). There are three symptom categories, namely (1) positive symptoms including hallucinations and delusions, (2) negative symptoms such as apathy and anhedonia, and (3) cognitive dysfunction and abnormal social behavior (Brennand et al., 2011). Currently, diagnosing SCZ is based on the symptoms and signs of psychosis.

A large number of susceptibility loci have been recently identified, among others dysbindin (DTNBP1), disrupted in schizophrenia 1 (DISC1), dopamine-catabolizing enzyme COMT, D-amino acid oxidase (DAO) and its activator (DAOA), glutamate decarboxylase 1 and 2 (GAD1/2), NRGNs, and NRXNs (Kirov et al., 2008; Robicsek et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics, 2014; Soares et al., 2011; Walsh et al., 2008).

There is increasing evidence from neural imaging, genetic analysis, post-mortem morphological, and pharmacological studies that altered DNA methylation and environmental stressors like cannabis are linked to SCZ (Grayson and Guidotti, 2013). Oligodendrocyte dysfunction and/or myelin deficits have been correlated to SCZ (Iwamoto et al., 2005). Accordingly, abnormal function of astrocytes or microglia have been associated with SCZ as well (Frick et al., 2013; Katsel et al., 2011). One of the first neurodevelopmental disease-specific stem cell model was established for SCZ by the reprogramming of human fibroblasts from four members of a family carrying a DISC1 variation (Wen et al., 2014). Neurons carrying the DISC1 variation revealed defects in neuronal connectivity, reduced outgrowth from soma, reduced Discs large homolog 4 (DLG4, also termed as PSD95) dendritic protein levels and altered downstream gene expression. Neural

progenitor cells derived from iPS cells of SCZ patients recently showed aberrant cell migration and increased oxidative stress (Brennand et al., 2015). Another group verified an increased level of reactive oxygen species in neural progenitors generated from SCZ patient-derived iPS cells (Paulsen Bda et al., 2012). The mood stabilizer Valproic acid altered the abnormal extra-mitochondrial oxygen consumption and reactive oxygen species to normal levels, which recapitulates the function of Valproic acid in a SCZ-specific stem cell model. The generation of SCZ patient-derived iPS cells was recently described by the reprogramming of hair follicle cells as a donor cell type (Robicsek et al., 2013). SCZ patient-derived neural progenitors were affected during differentiation into dopaminergic neurons and showed maturation defects of glutamatergic neurons. More recently, a SCZ-specific stem cell model for heterozygous mutations in the NRXN1 gene have been shown to cause functional defects on synapsis and neurotransmitter release (Pak et al., 2015). Drug screening for the analysis of valporate recently demonstrated positive effects on the potassium imbalance in SCZ-specific reprogrammed cells (Paulsen Bda et al., 2014).

Albeit iPS cell-based *in vitro* models have been described, major signaling pathways suitable for drug development has not been emerged highlighting the need for more suitable SCZ-specific disease models.

Taken together, promising disease-specific stem cell models have been developed for the analysis of neurodegenerative and neurodevelopmental diseases as well. However, it will be necessary to broaden the diversity of models and to improve available techniques suitable for HTS.

7.3 Stem Cell Models for Cardiotoxicity and Cardiovascular Disorders

Cardiac tissue generated from human pluripotent stem cells has attracted much interest for two reasons: (1) According to the WHO, cardiovascular disease is the leading cause of death worldwide (www.who.int/mediacentre/factsheets/fs317/en/, accessed February 28, 2016). Thus, creating a physiologically functional human myocardium would immensely broaden the spectrum of translational applications. (2) During preclinical and clinical drug development cardiotoxicity is the major cause of drug failures leading to post-approval withdrawal of medicines (Ferri et al., 2013). Approximately one-third of all safety-based pharmaceutical withdrawals are due to cardiotoxicity (Mathur et al., 2015). Current *in vitro* and *in vivo* cardiotoxicity and arrhythmogenesis models rely on animal- or tumor-derived cell lines, lines immortalized by genetic modification or perfused animal hearts (Mordwinkin et al., 2013). However, commonly used Chinese hamster ovary (CHO) cells or human embryonic kidney 293 (HEK293) cells that ectopically express a human cardiac ion channel do not necessarily recapitulate the function of a human cardiomyocte (Liang et al., 2013) and animal models are often not suitable due to inter-species variations. This is particularly highlighted in the mouse whose beat rate for instance is about 10 times faster than that of humans (Ho et al., 2011). In addition, increases in the heart rate in humans are correlated with increased force of contractions but decreased force in mice (Nerbonne, 2004). Ion channels driving repolarization are different in mice and humans as well (Salama and London, 2007). Issues also arise using rats and dogs which for example tolerate up to 100-fold of the concentrations of various chemotherapeutic agents than humans (Price et al., 2008).

7.3.1 Generating Cardiomyocytes *In Vitro*

One of the first approaches to generate cardiomyocytes from pluripotent stem cells used 3D aggregates, the so-called embryoid bodies, formed by ES cells in serum-containing medium. It has been well established with mouse (Doetschman et al., 1985; Van Laake et al., 2005). Such protocol using embryoid body-based cardiac differentiation of D3 mouse ES cells is still used for embryotoxicity screening called the embryonic stem cell test (EST). It was published as a standard operating procedure (Seiler and Spielmann, 2011), successfully validated by the European Centre for the Validation of Alternative Methods, and is widely

used in pharmacology (Buesen et al., 2009). The embryoid body approach was also tested using human pluripotent stem cells, but the initial yield was only between 5–15% (Kehat et al., 2001). Nowadays, differentiation protocols using human ES cells in monolayer cultures have essentially replaced the embryoid body formation (Kadari et al., 2015; Lian et al., 2013) and the use of small molecules and serum-free conditions make these protocols highly reliable and efficient leading to a yield of more than 90% cardiomyocytes.

7.3.2 Generating Microphysiological Systems to Mimic the Human Heart

Even though the aforementioned protocols generate cardiomyocytes in high quantities, an ideal *in vitro* model would mimic the *in vivo* environment of the heart as much as possible. This means taking into account the microenvironment geometry, cell-cell interactions, extracellular matrix composition and all this in a highly accessible system that is easily controllable. Topographical alignment of cardiomyocytes facilitating cell-cell interaction can be achieved using micro-fabricated nanostructured surfaces, thermoplastic shrink film, microcontact printing of cardiomyocyte patterns, or plasma-etched polyethylene glycol (PEG) surfaces (Mathur et al., 2016). These techniques can be combined with extra cellular matrix components such as laminins or fibronectin. Such systems have been taken to a higher level recreating organoid heart structures in a cardiac microphysiological system that allows recapitulating the geometry of perimysial collagen fibers, 3D alignment, microcirculation of nutrients, and removal of metabolic waste as well as shear flow protection (Mathur et al., 2015). Nowadays, the "heart-on-a-chip" technology allows intricate simultaneous analyses of the contractile function, quantification of the electrical propagation and evaluation of changes in the cytoskeletal architecture in a high throughput manner (Agarwal et al., 2013).

7.3.3 Disease-Modeling using Microphysiological Cardiac Systems

Ever since the iPS technology has been introduced (see Section 7.2.1), large efforts have been made to model cardiac diseases using iPS cells from patients with the LEOPARD syndrome (Carvajal-Vergara et al., 2010), long QT (Wang et al., 2014b), familial dilated cardiomyopathy (DCM) (Wyles et al., 2016), familial hypertrophic cardiomyopathy (HCM) (Han et al., 2014), and channelopathies such as the ones caused by mutations in potassium channels (Lahti et al., 2012). Further, iPS cell-derived cells have been used in conjunction with the aforementioned microphysiologcial cell systems. For instance, a mutation of the gene encoding tafazzin (TAZ) leading to a mitochondrial cardiomyopathy, the Barth Syndrome (BTHS), was examined by generating iPS cell lines from two patients with BTHS. The iPS cell-generated cardiomyocytes displayed sparse and irregular sarcomeres and engineered BTHS "heart-on-a-chip" tissues contracted weakly. Genome editing unequivocally linked the TAZ mutation to the observed phenotype (Wang et al., 2014a).

Taken together, heart tissue engineering has come a long way: microsystems nowadays provide cell and environmental interactions and functional readouts of force and/or electrophysiology on a high throughput basis. Therefore, they serve as valuable alternatives to animal models. Combining these microsystems with the iPS technology will allow us to gain deeper insight into the impact of mutation-based cardiac diseases. Therefore, even though these systems require further evaluation, they may replace the currently used screening methods in the foreseeable future.

7.4 Stem Cell Models for Toxicological Screenings of EDCs

Endocrine disruption is a major cause for obesity, which is linked to diabetes and cardiovascular problems. Endocrine-disrupting chemicals (EDCs) have been hypothesized to contribute to the high prevalence of diseases such as obesity, hypertension, neurodevelopmental disorders, and diabetes mellitus (Grun and Blumberg,

2009; Heindel and vom Saal, 2009; Latini et al., 2010; Testa et al., 2012). EDC exposure occurs during all phases of life. Early EDC exposure in the developing embryo may cause permanent metabolic alterations, thus potentially contributing to development of diseases later in life (Fowler et al., 2012). EDCs are polychlorinated biphenyls (PCBs), dioxins, and phthalates (such as DEHP). The plasticizer di(2-ethylhexyl) phthalate (DEHP) was commonly used in a wide range of products including food packages and medical devices (Hauser and Calafat, 2005). Because of its potential adverse effects on human health, the European Union banned the use of DEHP in children's products in 2005 (Directive 2005/84/EC, European Union 2005). Labeling of DEHP-containing medical devices is mandatory since 2007 in Europe. Today, multiple adverse effects of DEHP have been described and its use is subject to approval within the European Union (since 2015).

7.4.1 *In Vitro* Analysis of EDCs in Reproduction and Development

It is clear that multiple chemical classes act via multiple mechanisms to perturb normal early developmental processes. The effects may be additive and/or synergistic. Chemical mixtures strongly increase the complexity of mechanisms. EDCs have been described as potential risk factors for the reproductive health. DEHP and its metabolites are known to cross the placenta and reach the fetus (Adibi et al., 2008; Wittassek et al., 2009). Maternal DEHP exposure has been associated with impaired gonadal development and fertility in human males (Hauser et al., 2007; Pant et al., 2008; Sharpe, 2006; Swan, 2008). Phthalates have been described to affect the developing embryo, but molecular or cellular mechanisms have been poorly described and the analysis of phthalates has been predominantly performed in animal models or animal derived cells or tissues (Kay et al., 2013). Stem cell-based *in vitro* models provide the opportunity to analyze adverse effects of EDCs in a human system mimicking aspects of the human development.

Effects on the nervous system have been studied. EDC exposure has been shown to influence essential signaling pathways for the development of the CNS. Estrogen signaling is influenced by EDCs, and lower levels of estrogen have been linked to symptoms comparable with symptoms observed in SCZ patients (Huber et al., 2004; Segal et al., 2007). Disturbed estrogen function may also confer the risk for genetic mutations such as the Turner's syndrome, which contributes to the development of psychotic disorders such as SCZ (Prior et al., 2000). There are similarities between the neurotoxicological and behavioral pathology associated with exposure to bisphenol-A (BPA) and the abnormalities observed in SCZ (Brown, 2009). *In vivo* experiments reported adverse effects of EDCs on the cardiovascular system (Posnack, 2014). There are *in vitro* studies performed in chicken, rat and human cells providing data that DEHP exposure affects the differentiation and the functionality of cardiomyocytes. Recently, murine ES cells were applied within the EST describing the disturbed differentiation of cardiomyocytes in the presence of MEHP (Schulpen et al., 2013). DEHP and its metabolite mono(2-ethylhexyl) phthalate (MEHP) exert metabolic effects by activation of peroxisome proliferator-activated receptors (PPARs) α and γ, key mediators of lipid metabolism and adipogenesis (Feige et al., 2007; Lapinskas et al., 2005; Schmidt et al., 2012). Recent cell culture studies on 3T3-L1 preadipocytes showed a promotion of adipogenesis by MEHP (Feige et al., 2007). Adverse effects on the adipogenic differentiation were also described in mouse ES and mesenchymal stem cells (Biemann et al., 2012). Human stem cell models for the toxicological screening of phthalates aiming at the evaluation of their effects so far have been scarce. The generation of iPS cells has been described from bovine testicular cells (Wang et al., 2013). Androgen receptor-mediated apoptosis was demonstrated in response to phthalate exposure. Analysis of phthalate exposure in the bovine system revealed that iPS cells are more resistant to apoptosis than testicular cells (Lin et al., 2014).

7.4.2 *In Vitro* Analysis and Toxicological Screenings of Drugs

Besides modeling of disorders, pluripotent stem cells in general provide the opportunity to generate more functional and more suitable human *in vitro* systems for toxicological screenings. Experimental models for the prediction of drug absorption and metabolism include screening protocols in human primary hepatic cells and human Caco-2 epithelial colorectal adenocarcinoma cells.

Cytochrome P450 is a family of isozymes necessary for the biotransformation of several drugs. Therefore, drugs are analyzed in toxicological screenings whether they act as enzyme substrates, inducers, or inhibitors of cytochrome P450 to prevent adverse effects. For example, clozapine, which is an effective SCZ drug, is a substrate of CYP1A2 (Ogu and Maxa, 2000). EDCs are thought to alter the activity of CYP 450 affecting steroid synthesis (Maqbool et al., 2016). *In vitro* identification of chemicals, which induce cytochrome P450, is almost exclusively performed in primary human hepatocyte cell models. However, human hepatocytes have been generated from iPS cells by the application of differentiation models suitable for the robust induction of CYP isozymes (Ishikawa et al., 2015; Nakamori et al., 2016; Rahimi et al., 2015). Even though several hepatic differentiation protocols show the expression of CYP isozymes, human primary hepatocytes still provide the more relevant model for the examination of drugs (monitored as increase in mRNA expression or increased activity of CYP isozymes).

The widely applied enterocyte model of Caco-2 cells has difficulties in the evaluation of drug absorption and drug metabolism because the expression levels of genes involved differ from the expression levels observed in the humans (Ozawa et al., 2015). Ozawa and co-authors recently published an iPS cell-based model for the differentiation of enterocytes yielding higher levels of CYP3A4 and Peptide transporter 1 (PEPT1, officially termed as SLC15A1) in comparison to Caco-2 cells.

The application of iPS cells provides the opportunity to analyze genetic variations in response to drugs or chemicals, because the efficiency of drugs differs between patients depending on their age, sex, and many other factors. There is evidence that the patient-specific genetic background also alters drug efficacy. SNPs in drug-metabolizing genes were linked to different efficacy and toxicity (Amani et al., 2008; Evans and McLeod, 2003) highlighting patient-tailored treatment.

In conclusion, currently available iPS cell techniques and iPS cell models provide several *in vitro* models suitable for disease modeling of psychiatric diseases and CVD. The application of iPS cells for drug developmental, especially for drug development by the analysis of iPS cell-based disease models, was recently reviewed for a variety of diseases including several neurological disorders (Avior et al., 2016). However, there is a need to improve the characterization of the generated phenotypes analyzed in response to the manipulation of molecular drug targets, especially for HTS. Further, iPS cell models also provide promising *in vitro* models for toxicological screenings of pharmaceuticals and potentially dangerous chemicals or other adverse environmental factors as well.

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8

Three-Dimensional Culture Systems and Humanized Liver Models Using Hepatic Stem Cells for Enhanced Toxicity Assessment

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8.1 Introduction

The liver is the second largest organ in the body and is critical for maintaining normal physiological functions (Ramadori, et al. 2008). The liver is a complex bioreactor regulating the uptake, storage, and release of peptides, amino acids, lipids, carbohydrates, and vitamins (Godoy, et al. 2013). Numerous xenobiotics could exert toxic effects, and the liver can convert substances into toxic products or to exposed to hepatotoxic compounds (Groneberg, et al. 2002). With the rise of various metabolic disorders, the role of the liver has increasingly come into focus in the evaluation of drug metabolism and the toxicity of various substances.

The gold standard approach for toxicity testing involves complex *in vivo* studies, which are time consuming and costly (Soldatow, et al. 2013). With concerns about animal welfare, time and cost constraints, as well as the increasing number of chemicals requiring testing, the use of animals for experimental purposes has been widely criticized (Cosmides, et al. 1991). Some research groups have reported that the response observed *in vitro* is often distinct from the one observed *in vivo*. Preclinical animal studies are inadequate to evaluate toxicity due to species-specific variations between human and animal hepatocellular function, necessitating supplementation of animal data with additional assays to assess the human response.

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In vitro methods are routinely used in industry and by regulatory bodies for toxicity testing safety assessment, and risk evaluation, and these offer unique advantages. The greatest use of *in vitro* methods, however, is for elucidating toxicity mechanisms and investigating the biological processes involved in mediating toxic responses to xenobiotics and drugs. To better understand drug metabolic pathways and liver toxicity, the establishment of reliable *in vitro* model systems remains a key challenge. Since the liver, particularly its hepatocytes, is the main site of drug-induced toxicity in the body, considerable attention has been placed on the establishment of *in vitro* hepatic models. A vast spectrum of *in vitro* models has been developed during the past decades, including human liver slices, human liver cytosolic fractions, human liver microsomes, isolated perfused livers, primary human hepatocytes, and human cell lines (Brandon, et al. 2003, Guo, et al. 2011). The general advantage of those models for toxicity studies is reduced complexity. Liver slices retain cytoarchitecture *in vivo* but are only viable for several hours and are not amenable to high-throughput analyses (Khetani and Bhatia 2008). Microsomes are used in high-throughput systems to identify enzymes specific to drug metabolism (Khetani and Bhatia 2008) but lack the dynamic gene expression and intact cellular machinery necessary for meaningful toxicity testing. Low expression of drug-metabolizing enzymes and the lack of supporting cells necessary to form a cellular community consisting of liver sinusoidal endothelial cells, hepatic stellate cells, and Kupffer cells, are among the main disadvantages of these various systems.

8.2 Hepatic Cell Lines and Primary Human Hepatocytes

Hepatic cancer cell lines such as HepG2 show unlimited availability and maintain certain liver-specific functions (e.g. albumin production) but lack other important liver cell features, particularly drug metabolizing capacity (Vermeir, et al. 2005). Thus, use of cell lines can potentially lead to inaccurate estimation of drug toxicity if the drug is metabolized in a specific way *in vivo*. However, for certain purposes such as parent compound toxicity, the investigation of drug-induced metabolome effects, cell polarity or chemotherapy resistance, it was shown that the HepG2 cell line is a suitable model (Mueller 2013). Hep2/C3A is a clonal derivative of the HepG2 cell line and it was selected for improved differentiated hepatic phenotype. The newly established HepaRG cell line displays useful characteristics, including an ability to maintain cytochrome P450 (CYP450) activity for up to 3–4 weeks post-differentiation. The HepaRG cell line consists of hepatocyte-like and biliary cells in a 1:1 ratio. These hepatocyte-like cells express phase II enzymes and hepatic membrane transporters, indicating that this cell line could serve as a surrogate for primary hepatocytes (Hart, et al. 2010).

The limitations of cell lines can be avoided by the use of primary human hepatocytes, especially those freshly derived from human liver tissue. These cells are generally considered as the gold standard *in vitro* model for drug metabolism and toxicity assessment. Primary human hepatocytes are typically isolated from freshly removed human livers that contain either primary or secondary tumors. These cells display *in vivo*-like drug metabolism capability and other key hepatic-specific functions such as carbohydrate metabolism, plasma protein synthesis, and CYP expression for several hours post-isolation; thus, primary human hepatocytes can be extensively used for toxicity studies (Sivaraman, et al. 2005). However, using present *in vitro* culture techniques, cell viability is limited, and hepatic dedifferentiation and loss of biotransformation capacity can occur within several days. Long-term culture is largely impeded by the progressive loss of the hepatic-specific phenotype at morphological and functional levels (Vinken, et al. 2012). These limitations influence the accuracy of toxicity studies and affect the reproducibility of results. Thus, primary human hepatocytes afford a limited time period for drug toxicity experiments. However, some reports described modified culture media capable of improving the long-term viability and functionality of hepatocytes, making chronic toxicity studies possible (Kostadinova, et al. 2013, Thurnherr, et al. 2011). Nevertheless, limited availability, donor shortage, and donor variability remain important technical obstacles for *in vitro* hepatotoxicity studies. Alternative sources of functional hepatocytes are urgently needed.

8.3 Embryonic Stem Cells and Induced Pluripotent Stem-Cell Derived Hepatocytes

Primary human hepatocytes are commonly used for *in vitro* toxicity studies. However, the use of non-transplantable livers is costly, there is an inadequate supply, and cells vary greatly between laboratories and protocols. Instead of fresh hepatocytes, the use of cryopreserved hepatocytes is becoming more popular as a substitute. However, cell quality upon thawing is not always stable. A more robust and reproducible source of hepatocytes would greatly benefit the field of toxicity testing and assist in standardizing research protocols. Theoretically, stem cells would represent a renewable source of cells and would potentially provide large numbers of functionally equivalent cells that could be stored for later use. A toxicity model that incorporates a relatively unlimited supply of human hepatocytes with a defined phenotype, would allow for improved reproducibility in evaluating drug responses and could account for the genetic diversity within the human population.

Human embryonic stem cells (hESCs) are isolated from the inner cell mass of a blastocyst and can give rise to all three embryonic germ layers (endoderm, mesoderm, ectoderm) (Thomson, et al. 1998). These cells maintain their undifferentiated state over many passages (i.e., they are self-renewable) and can be differentiated into all three germ layers, both *in vitro* and *in vivo* (Agarwal, et al. 2008). hESCs have enormous potential as a source for cell replacement therapies and as a model for early human development (Baharvand, et al. 2006). Many laboratories have developed protocols to isolate hESCs and induce the formation of hepatocyte-like cells (Baharvand, et al. 2008, Duan, et al. 2007, Hu, et al. 2010, Zhao, et al. 2009). Multiple hESCs differentiated into the hepatic lineage showed detectable levels of many CYPs, although CYP2C9, CYP2C19, CYP2D6, and CYP3A4 were undetectable, and fetal CYP3A7 was highly expressed, suggesting an immature phenotype (Ek, et al. 2007). Hamazaki *et al*. (Hamazaki, et al. 2001) evaluated the capability of murine embryonic stem cells to differentiate into mature adult hepatocytes *in vitro*. Duan *et al*. (2010) showed that most CYP proteins were expressed in several batches of hESC-hepatocytes. Albumin and α1-antitrypsin gene expression levels were 75% and 64% that of freshly isolated primary human hepatocytes, respectively. Moreover, liquid chromatography/mass spectrometry analysis revealed that phase-II conjugation metabolites of bufuralol in hESC-hepatocytes and primary human hepatocytes were identical. However, another group described hESC-hepatocytes that had significantly lower CYP3A4 activity (most abundant CYP in the liver) with a 1000-fold reduction in *CYP3A4* transcripts relative to that of primary human hepatocytes (Wobus and Löser 2011). The potential differences in the maturation status of the different cell lines and different differentiation protocols cannot be entirely ruled out to explain the variable findings of the aforementioned studies; cells begin to lose hepatic characteristics after a few days (Medine, et al. 2013). Nonetheless, despite having high alpha-fetoprotein (fetal marker) expression, hESC-hepatocytes treated with rifampin showed an induction of CYP3A4 activity; further maturation of hESC-hepatocytes towards an adult liver phenotype is needed (Davidson, et al. 2015). Researchers are currently trying to modify methods to overcome these disadvantages, which include functional variability, low expression levels, and loss of functionality over time. However, hESCs are a controversial cell source for scientific research and that is not likely to change in the near future. A different source of stem cells could eliminate this ethical controversy (Soldatow, et al. 2013).

Human ESCs could provide a source of hepatocytes suitable for cell transplantation and a useful model to study drug toxicity (Agarwal, et al. 2008). However, ethical concerns around the destruction of embryos have led to significant limitations in the generation of new hESCs using U.S. federal funding (Davidson, et al. 2015). Recent advances in the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) by forced expression of four defined transcription factors through viral transfection offer a nearly unlimited supply of stem cells without the ethical concerns associated with hESCs (Takahashi, et al. 2007, Yu, et al. 2007). iPSC technology brings together the potential benefits of hESCs (i.e., self-renewal capacity and pluripotency) and addresses major ethical and scientific concerns of hESCs. Use of iPSCs bypasses the ethical concerns of embryo destruction since they are produced from somatic cells *in vitro* without embryonic

tissues or oocytes; use of these cells also addresses immune-compatibility issues, since they are generated from patients with inherited metabolic conditions and may be used as liver disease models (Yu, et al. 2014). More recently, iPSCs have been produced without viral vectors and transgene sequences using non-integrating episomal vectors to decrease the risk of tumorigenicity for applications in regenerative medicine (Okita, et al. 2008, Yu, et al. 2009). With the advent of footprint-free episomal primary tissue reprogramming to iPSCs, iPSCs with easy accessibility provide new hope for *in vitro* disease modeling and drug toxicity assessment (Soldatow, et al. 2013). It was shown that hepatocyte-like cells (HLCs) could be generated from iPSCs. Our previous study (Takebe, et al. 2013) and multiple reports (Kajiwara, et al. 2012, Si-Tayeb, et al. 2010) of the potential benefits of HLCs generated from human iPSCs have described their secretion of human albumin, alpha-1-antitrypsin (A1AT), and hepatocyte nuclear factor 4-alpha (HNF4α), urea synthesis, and expression of cytochrome P450 (CYP) enzymes *in vitro*. However, iPSC-HLCs showed expression levels of xenobiotic metabolism genes that were not equivalent to those found in whole liver or freshly isolated primary hepatocytes, and the CYP enzyme levels decreased quickly over the culture period (Gerbal-Chaloin, et al. 2014, Soldatow, et al. 2013). However, cell line variation and incomplete reprogramming continue to impede progress towards the application of these cells for *in vitro* toxicology studies (Schwartz, et al. 2014, Si-Tayeb, et al. 2010). Some reports also found that the origin of iPSCs could influence their differentiation, tumorigenic properties, and gene expression and epigenetic features (Marchetto, et al. 2009, Miura, et al. 2009).

While the application of iPSC-HLCs in toxicity testing represents a paradigm shift for *in vitro* drug development, these systems are not perfect. Due to current disadvantages and technical limitations, neither induced pluripotent nor embryonic stem cells are yet a widely accepted option for toxicological and pharmacological studies. Future advancements in culture systems will certainly serve to further elevate iPSC-HLCs as a model for predictive toxicity evaluation that represents a broad patient population.

8.4 *Ex Vivo*: Three-Dimensional and Multiple-Cell Culture System

In vivo, nearly all cells in tissues reside in an extracellular matrix (ECM) consisting of a complex three-dimensional (3D) architecture and interact with cells nearby through biochemical and mechanical cues. Cell–cell and cell-to-extracellular interactions establish 3D communications that maintain tissue specificity and modulate cell function. Two-dimensional (2D) monolayer cell cultures have played a vital role in the study of developmental biology, drug discovery, drug toxicity, and regenerative medicine. However, 2D culture-derived cells are unable to achieve *in vivo*-like structural organization; these cells display limited or diminished cellular morphology, proliferation, differentiation, as well as gene and protein expression related to drug metabolism and cell function (Bissell, et al. 2002). To overcome some of these limitations, numerous 3D cell culture models have been developed over the past two decades. Several of these studies have demonstrated that when cells are grown in 3D environments, they express a number of physiological characteristics that more closely resemble those of the native tissue from which they originated than that of the same cells grown in a traditional 2D culture system (Fey and Wrzesinski 2012, Gieseck, et al. 2014, Gunness, et al. 2013). These findings are potentially associated with the fact that a 3D cell culture system enables establishment of physiological cell-cell and cell-ECM interactions that mimic the microenvironment of native tissue (Daus, et al. 2011, Lin, et al. 2006); this feature leads to a recovery or maintenance of *in vivo* function (Loessner, et al. 2010; Selden, et al. 2000). Previous studies have demonstrated that 3D culture prolongs maintenance of cytochrome P450 expression (Adachi, et al. 2011), enhances drug toxicity activities (Oshikata, et al. 2011), promotes expression of key hepatic markers spheroids using primary liver cells (Tostões, et al. 2012), and prolongs liver functionality (Bachmann, et al. 2015, Brophy, et al. 2009). 3D hepatocyte cultures show liver-like structures and functional activity comparable with those observed in the *in vivo* condition, and can therefore be used for dose-response studies (Abu-Absi, et al. 2002, Kostadinova, et al. 2013).

Although 3D sphere culture systems have made a great contribution in improving the understanding of liver cell function and drug toxicity *in vitro*, there are still obstacles that make quantitative toxicology using 3D cultures more complicated than using 2D cultures. These limitations include difficulties associated with cell count and abnormalities associated with cell viability and proliferation control due to the particulate status of 3D cultures (Fey and Wrzesinski 2012).

In vitro liver cells quickly lose their morphology and liver-specific function, even under standard culture conditions (Shulman and Nahmias 2013). Several recent studies have demonstrated the preservation of cell structure and function for up to several months using an *in vitro* culture system featuring co-culture with different cell types (Baxter, et al. 2010, Bhatia, et al. 1999, Nelson, et al. 2015), consistent with the effects of iPSC on activated mouse embryonic feeder cells (Thomson, et al. 1998). The liver is comprised of hepatocytes and non-parenchymal cells, including Kupffer cells, stellate cells, and endothelial cells (Ballet, et al. 1984). Co-culture of liver cells with liver-derived or non-liver-derived stromal cells have been reported to stabilize the liver phenotype, improve cell survival, and maintain cell function, both in human liver cells and animal liver cells (Abu-Absi, et al. 2004, Krause, et al. 2009, Liu, et al. 2014a, Shulman and Nahmias 2013). Examples of co-culturing hepatocytes with different cell types such as human umbilical vein endothelial cells (Lecled, et al. 2003, Nelson, et al. 2015, Salerno, et al. 2011, Takebe, et al. 2013), fibroblasts (Cho, et al. 2008, Liu, et al. 2014a,b), and different non-parenchymal liver cells (Bale, et al. 2014, Hwa, et al. 2007, Kim and Rajagopalan 2010) showed maintenance of cell functionality of all cell types, including biotransformation capacity of hepatocytes, synthetic function, mRNA expression, CYP450 activity, and drug clearance potential (Shulman and Nahmias 2013). However, these co-culture methods have a high lab-to-lab variability and they are not aligned with the technical requirements of the pharmaceutical industry, as they are not optimized for high-throughput analyses.

8.5 *In Vivo*: Humanized Liver Models

One of the main obstacles of drug toxicity and metabolism studies is the quantitative prediction of *in vivo* drug metabolism based on *in vitro* data (Lin and Lu 1997). *In vivo* drug testing in animal species has not always accurately predicted human drug metabolism for candidate drugs (Andersen and Krewski 2009), since interspecies differences in drug metabolism result in different metabolite profiles (Nishimura, et al. 2013). Thus, it is challenging to identify human-specific drug metabolites, as it is most often a drug metabolite, and not the parent drug, that is responsible for an unexpected drug-induced toxicity (Bale, et al. 2014). To solve this problem, our group (Tsuchida, et al. 2014, Zhang, et al. 2015) and others (Azuma, et al. 2007, Hasegawa, et al. 2011, Suemizu, et al. 2008, Tateno, et al. 2004) have developed chimeric mice and rats, in which the animal livers are replaced by transplanted human liver cells or tissue-engineered human liver tissue (Chen et al., 2011). These rodent hosts enable human liver cells to proliferate and engraft to repopulate the damaged rodent liver, where they can then synthesize human proteins, CYP450s, and mediate human drug biotransformation reactions (Peltz 2013). Previous studies have described mouse models used for chimeric mouse generation, including a uroplasminogen activator transgene expression model to facilitate the growth of transplanted human liver cells (Katoh, et al. 2004, 2005), a fumarylacetoacetate hydrolase knockout mouse (Azuma, et al. 2007, Bissig, et al. 2010), a thymidine kinase transgene (TK-NOG) mouse (Hasegawa, et al. 2011), and our recently reported Alb-TRECK/SCID mouse (Zhang, et al. 2015). The humanized liver of chimeric Alb-TRECK/SCID mice was shown to express mRNAs encoding human cytochrome P450 (P450) enzymes, transporters, and transcription factors affecting drug metabolism at levels that were equivalent to those in human adult hepatocytes. Moreover, there was extensive human CYP3A4 protein expression in humanized livers, and chimeric Alb-TRECK/SCID mice could mediate human-specific drug biotransformation reactions (Zhang, et al. 2015).

Some studies described chimeric mice that produced known human-specific metabolites for several test substrates (Xu, et al. 2014, Zhang, et al. 2015), which could be used to predict human drug metabolism of a candidate therapeutic before human clinical testing. The use of humanized mice could enable human-predominant drug metabolites to be identified before performing human drug exposure studies (Strom, et al. 2010). Therefore, toxicology studies using chimeric mice and rats could have a large impact on drug development and could improve the safety of drugs that will be subsequently tested in humans. Moreover, we have previously demonstrated that the rate of drug metabolism in chimeric mice can be selectively regulated by the transplantation of hepatocytes obtained from human donors with different alleles in genes affecting drug metabolism (Zhang, et al. 2015). It is also important to consider the current limitations of using chimeric mice for toxicology testing. Since they are highly immunocompromised, chimeric mice cannot be used to analyze immune-mediated drug toxicities. We hope that the information presented here will lead to the broader use of chimeric animals in preclinical toxicology studies, since the application of modern methodology could improve the safety aspects of drug development.

8.6 Summary

Primary human hepatocytes and immortal cell lines are commonly used to assess chemical toxicity. However, the limited stability and insufficient supply of primary cells, as well as validation concerns are still unresolved. Embryonic stem cells and induced pluripotent stem cell-derived hepatocytes are potentially more attractive for application in industrial settings and preclinical purposes due to their unlimited expansion capacity. However, differentiation of these cells into mature hepatocytes is incomplete, and a supportive microenvironment is needed to accurately promote the developmental process with a structured multiple-cell society that includes liver sinusoid endothelial cells, stellate cells, Kupffer cells, and hematopoietic cells. It remains a challenge to design useful studies that accurately reflect the *in vivo* situation in humans and that are reproducible. To mimic and create an *in vivo* environment in rodents, humanized liver models of mice and rats are developing using proliferative hepatic cells from embryonic stem cells and induced pluripotent stem cells or other somatic stem cell populations. The broader application of *ex vivo* multiple-cell systems and *in vivo* humanized animals would potentially increase the quality of toxicity studies and preclinical evaluations.

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9

Utilization of *In Vitro* Neurotoxicity Models in Pre-Clinical Toxicity Assessment

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9.1 Introduction

9.1.1 Limitations of Animal Models and the Utility of *In Vitro* Assays for Neurotoxicity Testing

Neurotoxicity is one of the major causes for adverse drug reactions (ADR) and drug attrition during pre-clinical or clinical development together with cardiovascular and hepatic related toxicities (Kola and Landis 2004; Valentin and Hammond 2008). Drugs being developed for central nervous system (CNS) therapeutic indications have some of the lowest success rates from first-in-man to registration (Kola and Landis 2004) and approximately 10% of all drug withdrawals, regardless of therapeutic area, were caused by neurospecific toxicities between 1960–1999. One big discrepancy that Astra Zeneca identified in a recent review of their portfolio was the difference in the magnitude of terminated programs due to CNS toxicity in pre-clinical animal models (7%) and the identification of clinical CNS toxicities resulting in safety failure (34%) (Cook 2014). The importance of refining risk assessment early in drug development is therefore highlighted in part by the high attrition rate and may reflect the limitations of current pre-clinical animal models, which fail to clearly identify the risks of neurotoxicity. The pharmaceutical industry currently relies heavily on animal testing to assess neurotoxicity associated with new drug candidates. The main objectives are to identify the risk for overt neurotoxicity and assess neurobehavioral effects through cage-side clinical observations, functional observational batteries (FOB), and histopathological endpoints (Hamdam et al. 2013). These assessments are often followed by more extensive evaluations if needed, such as electrophysiology and extended behavioral testing based on initial results (Fig. 9.1/Plate 2). For example, *in vivo* behavioral and neurological studies including neurophysiological recordings such as electroencephalogram (EEG), measurements of nerve conduction velocity, and neurochemical assessments (such as dialysis, biomarkers, and imaging) are

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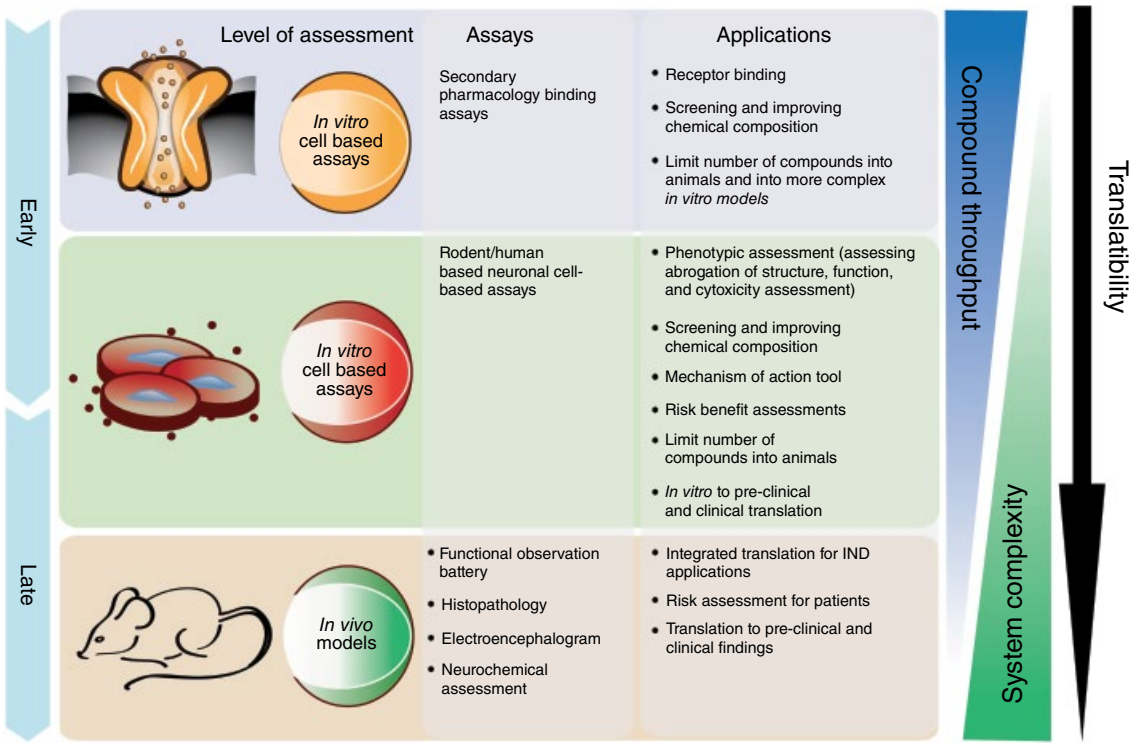

Figure 9.1 ***(Plate 2)*** *High level neurotoxicity evaluation cascade in drug development. Preliminary assessments of the potential for neurotoxicity using simple* in vitro *tools aim to improve chemical matter and reduce the number of compounds moving through the pipeline as potential drug candidates. Once promising candidates have been selected, additional risks (depending on the target), and the mechanism of toxicity observed both pre-clinically and clinically can be evaluated in neuronal specific cell assays to query functional effects. Ultimately, these neuronal specific* in vitro *models further reduce the number of compounds that are assessed in animal studies and help to identify the molecular underpinnings of neurotoxicity. This diagram presents approaches conducted both at the early and late phase of drug development process. (See insert for color representation of the figure.)*

sometimes used to assess and further characterize neurotoxicity. Together, these described methods may be utilized to assess the impact of a molecule on the CNS and peripheral nervous systems (PNS). It can query behavioral impact and potential influences on cognition, along with sensory and motor function (Hamdam et al. 2013) thereby making animal testing a preferred model for neurotoxicity assessment.

However, there are several limitations to utilizing animal models to characterize toxicity. *In vivo* models are impractical for screening large numbers of chemicals due to low throughput, high cost, and limited predictive value with regard to its translation to clinical findings (Bal-Price et al. 2011). Animal studies also raise ethical concerns (Bal-Price et al. 2011; Cook 2014; Dragunow 2008; Kola and Landis 2004; Schultz et al. 2015; Valentin 2008), may lack sensitivity in capturing safety endpoints, and may not adequately represent human-specific pathophysiology. Hence, the information obtained from animal models may not facilitate a comprehensive understanding of the molecular underpinnings of toxicity. Developmental and structural differences between human and rodent nervous systems suggest some underlying differences in their molecular and cellular events. Some examples include species differences in neurogenesis and electrophysiological properties of neurons. Certain regions are more developed in humans such as the prefrontal and temporal

cortex important in neuropsychiatric disorders (Allman et al. 2011; Clowry et al. 2010; Dolmetsch and Geschwind 2011; Dragunow 2008; Steffenhagen et al. 2011). Furthermore, rodents may not recapitulate toxicity that is due to a disease phenotype or more complex adverse effects such as suicidality observed with drugs such as, for example, rimonabant (Christensen 2007). These differences may therefore contribute to the poor translatability of pre-clinical studies. In light of the high drug attrition caused by neurotoxicity during drug development and the limitation of animal models, there is an urgent need to identify and develop alternative *in vitro* tools that could streamline the drug development process and potentially increase the safety of new drug candidates (Bal Price et al. 2011, 2010a,b, 2015; Coecke et al. 2006; Schultz et al. 2015). Hence, successful drug development processes would involve the implementation of complementary *in vitro* approaches that can be utilized to address specific mechanistic questions around theoretical or identified neuropharmacological effects, whether detected during the development process or after approval (Fig. 9.2/ Plate 3). This includes hypothetical risks based on data from the target or from previous compounds within the target class. In addition to providing mechanistic information and translatability assessment, such assays could also be used as screening tools to improve the chemical content and mitigate the potential neurotoxicity of future drug candidates. Screening assays could also help to limit the number of compounds that are tested *in vivo* thereby supporting the principle of 3Rs (reduce, replace, refine), as well as reducing the time and costs associated with neurotoxicity testing. Finally, once the models are appropriately characterized, the step-wise screening could reduce compound attrition in later stages of drug development due to previously undetected neurotoxicity risks. Achieving a comprehensive understanding of the risk-to-benefit relationship during early stages of drug development and the molecular underpinnings of the drug safety issues could help to limit the impact that safety issues may have on clinical use and withdrawal due to safety issues related to neurotoxicity (Bowes et al. 2012).

9.1.2 How Regulatory Requirements Can Shape the Development of *In Vitro* Screening Tools and Efforts

The use of *in vitro* models to predict neurotoxicity and to gather mechanistic insights to complement, animal studies has been proposed (Bal-Price 2010a,b, 2011, 2015; Coecke 2007; Collins 2008; Harry and Tiffany-Castiglioni 2005; Johnstone et al. 2010; Lein et al. 2005; Shultz 2015; Sison-Young et al. 2012). Less frequently, *in vitro* assays have been used as screening tools to study specific aspects of neurotoxicity (Johnstone et al. 2010). There has been recent work in the application of *in vitro* models to identify chemical-related developmental neurotoxicity (DNT) risk in humans (Bal-Price et al. 2010b; Schultz et al. 2015; Wilson et al. 2014). Many chemicals are known to impact neurodevelopmental processes and cause DNT. Regulatory agencies such as the U.S. Environmental Protection Agency (USEPA) and the Organisation for Economic Co-operation and Development (OECD) focus on pre-clinical animal studies to assess the risk for chemical impact on adult and developmental neurotoxicity (Bal-Price et al. 2010a). The REACH policy (Registration, Evaluation, Authorization, and Restriction of Chemicals, European Commission 2003) is a characteristic example to illustrate how a policy has influenced the efforts around developing alternative methods (both *in silico and in vitro)* to fulfill a requirement to assess more than 30,000 existing chemicals for neurotoxicity (Bal-Price et al. 2008, 2010a,b, 2011; Coecke et al. 2006, 2007, 2008; Harry and Tiffany-Castiglioni 2005). The use of such models would enable the evaluation of large sets of chemicals to fulfill the regulatory requirements. This initiative highlights the importance of ongoing efforts to establish relevant, high throughput screening (HTS) assays for adult and neurodevelopmental toxicity assessment (Bal-Price 2011, 2015; Coecke et al. 2006; Johnstone et al. 2010: Rovida et al. 2015; Schultz et al. 2015). In the pharmaceutical industry setting one can get insights from scientific efforts and discussions around environmental chemical testing to improve neurotoxicity hazard identification (ID) in drug development.

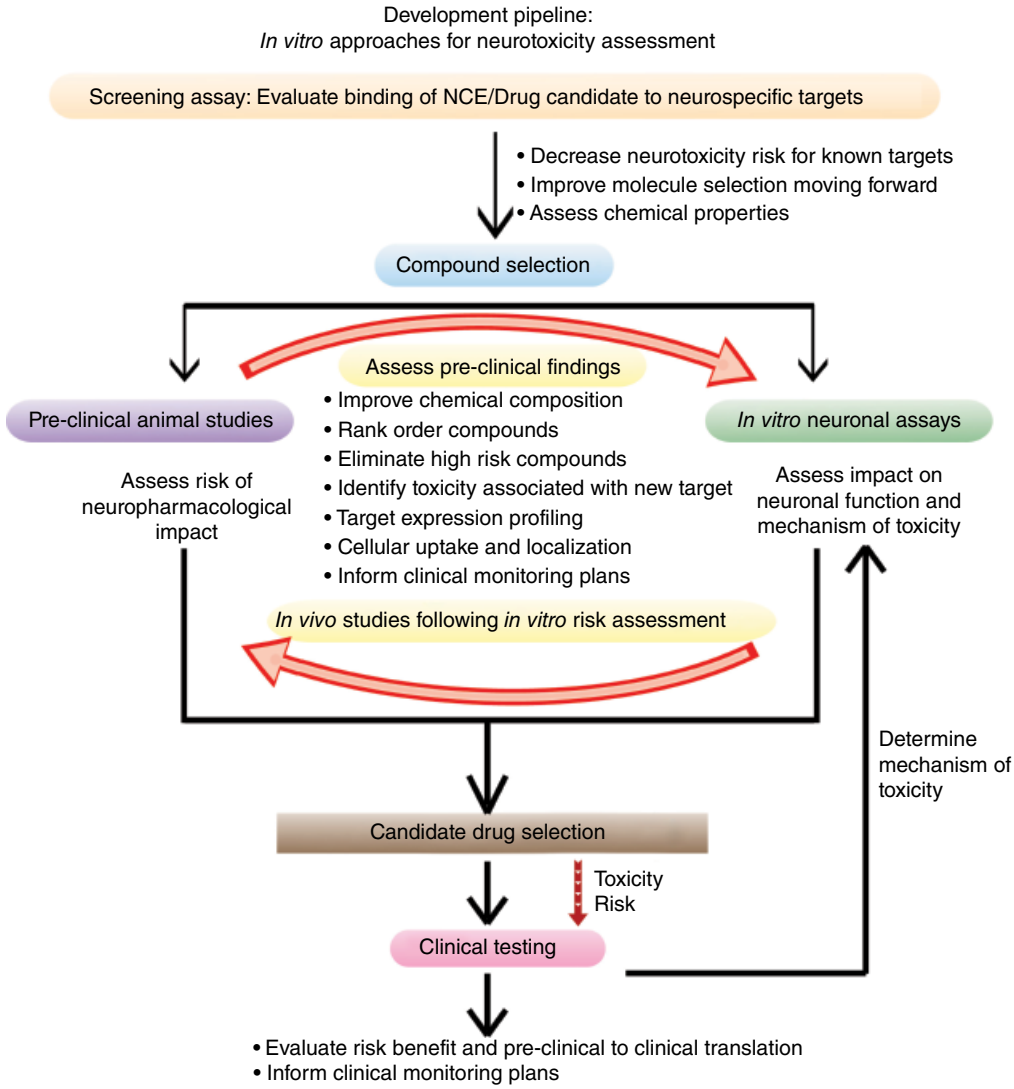

Figure 9.2 ***(Plate 3)*** *Flowchart illustrating the incorporation of* in vitro *approaches for neurotoxicity assessment in the drug development process and highlights the potential impact on drug candidate selection and neurotoxicity risk assessment aiming to reduce the clinical impact on attrition. (See insert for color representation of the figure.)*

9.1.3 *In Vitro* Assays as Useful Tools for Assessing Neurotoxicity in a Pharmaceutical Industry Setting

In vitro screening tools and models are steadily emerging to assess mechanism of neurotoxicity in an industry setting. Standard *in vitro* assessments for neurologically relevant targets incorporated early in the drug development process currently include biochemical and/or cellular binding studies (Fig. 9.1/Plate 2). These preliminary studies are usually performed using engineered cell lines to test on- or off-target

binding of important neurospecific receptors, enzymes, transporters, and ion channels. The rationale behind these assays is to test pharmacological targets that have been associated with clinical adverse effects or that could potentially have a major impact on physiological function (Bowes et al. 2012; Kramer et al. 2007; Whitebread et al. 2005). Currently, the receptor binding panels coupled with additional *in vitro* cell based functional agonist/antagonist assessments provide an early tool for investigating the potential for chemicals to bind important receptors and enzymes, and evaluate their neurological impact. These early risk assessments offer several advantages. They help to identify potential target liabilities, provide directions to chemists to assess structure-activity relationships, and aid in developing improved chemical libraries by selecting compounds exhibiting increased potency and potentially lower risk for neurotoxicity. The limitations of these assays are that they are artificial systems using recombinantly expressed proteins and the number of selected receptors and ion channels assessed is relatively small when considering the pharmacological target space. In addition, such models do not take into account the complexity of a neuronal cell and its environment *in vivo*. Hence, not all targets or cell types involved in neuronal systems are represented accurately. Neuronal specific cell-based *in vitro* assays implemented early in the drug development process could provide a more physiologically relevant screening tool to identify and prioritize molecules for *in vivo* testing (Fig. 9.1/Plate 2). These cell-based models could be applied towards understanding explicit mechanistic and functional effects of selected compounds on neurons.

Neurospecific functional *in vitro* assays are starting to be implemented in drug development for toxicity assessments of compounds where the target is either present in neuronal tissues or target regulation may affect the CNS or PNS. Additional assessment of compounds with chemical properties similar to known neurotoxic chemicals or drugs that have the potential to cross the blood brain barrier (BBB) may also be included for hazard identification in these types of neuronal specific assays. Functional cellular assays can be incorporated following observed neurotoxicity in pre-clinical studies or to help clarify mechanistic understanding of clinical findings (Fig. 9.2/Plate 3). Improved mechanistic understanding of a clinical finding can be helpful during initial stages of developing a potential clinical mitigation strategy or monitoring plan. It also aids decision-making regarding a chemical series and candidate selection. Benchmarking of in-house compounds against marketed drugs and/or molecules with known neurotoxicity effects enables the team to make informed decisions around risk benefit and dose selections. The *in vitro* platforms described previously are designed to provide utility both early and late in the drug development process, but are currently not used to make a final decision about the interpretation of a particular compound's toxicity. Rather, they add to the weight-of-evidence around a compound's toxicity and can also be used to compare molecules to each other to rank-order levels of neurotoxicity.

This chapter will review the *in vitro* neuronal cell models that are currently being developed and/or utilized as integrated tools to bridge the gap between pre-clinical drug development and clinical effects. We will focus on stem cell-derived models that have potential advantages to assess drug effects on neurons at the molecular, structural, or functional level in a pharmaceutical setting.

9.2 Current Models of Drug-Related Clinical Neuropathies and Effects on Electrophysiological Function

Some of the most critical concerns in drug development are to determine whether there is any risk for causing toxicity in the CNS or PNS. The risks could be due to functional effects alone or a combination of structural changes leading to functional effects. These effects include abuse liability, seizurogenic potential, depression or activation of the CNS, neurodegeneration (neuropathy), and neurodevelopmental impact.

9.2.1 Neuropathy Assessment

Several oncology treatments such as chemotherapeutics exhibit effects on the PNS that coincide with neuropathies that are often dose limiting toxicities in the clinic. Chemotherapy-induced peripheral neuropathy (CIPN) is associated with a multitude of oncology drugs including compounds targeting the microtubules (taxanes, vinca alkaloids, epothilones, maytansinoids, and dolastatins), proteasome-inhibiting compounds (bortezomib and thalidomide), or DNA-crosslinking compounds (platinum salts) (Balayssac et al. 2011; Dumontet and Jordan 2010; Grisold et al. 2012). Similar toxicities have been observed with antibody drug conjugates that employ a chemotherapeutic-based payload (Saber and Leighton 2015).

In the case of peripheral neuropathy, neurologic testing in animals is commonly used to assess effects on neurons, which includes histopathology of the sciatic nerve or dorsal root ganglia (DRG) (Hoke and Ray 2014; Poon et al. 2013). Animal models for neuropathy and neuropathic pain have provided value in basic research to try and understand the molecular underpinnings of neuropathy. However, due to inconsistency in animal models of CIPN induction along with the complex and differing molecular underpinnings by which chemotherapeutics act to induce neuropathy, its pathophysiology is not fully understood (Balayssac et al. 2011; Carozzi et al. 2010; Han et al. 2013; Hoke and Ray 2014; Jaggi and Singh 2012). While animal models can predict CIPN for some classes, they are less predictive for others (Authier et al. 2009; Carossi et al. 2010; Poon et al. 2013; Saber and Leighton 2015).

The *in vitro* endpoints most frequently used to measure the effects of chemicals on neurodevelopment and to mimic clinical neuropathy are neurite dynamic changes and viability. Developmental/neuro toxicity assessment in response to environmental chemicals has been previously addressed (Bal-Price et al. 2010a,b, 2011; Breier et al. 2010; Castiglioni 2006; Coecke et al. 2006, 2007; Radio et al. 2008, 2010) and will not be discussed in detail in this chapter. Clinical neuropathy is characterized most often by a "dying back" of distal sensory neurons along with motor involvement in some instances. Symptoms include tingling, numbness, pain, paresthesia, sensory ataxia, along with mechanical and cold allodynia (Sisignano et al. 2014). Many of the classical chemotherapeutics affecting the microtubule (such as vincristine and paclitaxel) are thought to disrupt axonal transport by affecting microtubule dynamics in addition to affecting respiration, mitochondrial function, and cell viability. In addition to causing direct neurite or neuronal effects, chemotherapeutics such as paclitaxel can also trigger astrocytes to activate inflammatory pathways (Zhang et al. 2012). The general pathology stemming from activation of glial and inflammatory cells is, however, not captured in simple two-dimensional (2D) neuronal cultures and requires co-cultures or tissue ex-plants. On the other hand, molecular events that can be successfully modeled *in vitro* include neurite dynamic changes, the binding and disruption of tubulin by biochemical assays, cell viability and effects on mitochondrial function. These endpoints can be used for assessing the underlying mechanism of toxicity with the potential to distinguish chemical classes, as well as rank order compounds based on toxicity and benchmarking those effects to known neurotoxicants for risk assessment purposes.

High content neuronal toxicity screening platforms utilizing primary cells such as neurons derived from embryonic stem cells (ESC), DRG, fetal tissues, for example cerebellar and cortical neurons, immortalized cell lines or induced pluripotent stem cell (iPSC)-derived neurons, and astrocytes are currently being developed by several companies and academic investigators (Efthymiou et al. 2014; Radio et al. 2010; Wilson et al. 2014). These high content screening or HCS assays used to assess neurotoxicity across neuronal cells, rely upon automated imaging devices that can identify cell specific phenotypes and often include a nuclear- or cytoplasmic-marker, as well as a cell specific marker (Anderl et al. 2009). These assays have the advantage of being able to measure a variety of endpoints per well and thus maximize the amount of information captured related to drug effects on these cells. The endpoints measured to assess clinical chemotherapy-induced neuropathy *in vitro* include impact on neurite dynamics such as neurite length and branch points. To identify neurite dynamic effects, high content assessment of changes in neuronal specific structural proteins,

such as MAP2 or BIIIi tubulin, are generally coupled with a nuclear stain for cell number and viability identification. Other cell health parameters are multiplexed on a hypothesis-driven basis, and may include mitochondrial function and metabolic activity. These approaches often involve terminal sampling of cells to stain and analyze cellular endpoints. Thus, repeated sampling to assess longitudinal changes is not commonly used and is a limitation of the described approaches.

9.2.2 Seizure Potential and Electrophysiological Function Assessments

Apart from neurodegeneration such as neuropathy described previously, other key effects on neurophysiology that disrupt neuronal function needs to be addressed in drug development. These effects include abuse liability, seizurogenic potential of a compound and risk for depression or activation of the CNS (Bassett et al. 2014; Dunlop et al. 2008; Hudzik and Markgraf 2015; Kalso and Simojoki 2014). This can manifest as a consequence of drugs and chemicals interacting with ion channels, enzymes, and receptors important for neurotransmitter release, synaptic function, and ion channel function. The early screening of compounds for receptor binding, which is included in drug discovery activities, can identify an increased risk for suicidality, abuse potential, or potential effects on electrophysiological function (increased or decreased activity) depending on what receptor is targeted (Muller et al. 2015). Following up with specific mechanistic studies in neuronal cell models or *ex vivo* tissues to further investigate the potential liability for a pharmacological mechanism can be done by using *in vitro* functional assessments. This includes investigating synaptic function, neurite changes, and electrophysiological changes. In addition, *in vivo* EEGs from scalp or intracranial recordings are useful in detecting CNS activation and depression including the detection of seizures indicative of epilepsy. Important functional assessments of electrophysiology and neuronal function have been evaluated extensively *in vivo*, have also been assessed in primary cell preparations, and *ex vivo* brain slices in culture (Johnstone et al. 2010). Brain slices have also been used to study neuronal electrophysiology and long-term changes in plasticity with the advantage of maintaining an intact cellular organization. Furthermore, rodent ESC, hippocampal brain slices, aggregating brain cultures, fetal cortical neuronal networks, and iPSCs have successfully demonstrated electrical activity *in vitro* and their utility in the assessment of neuroactive substances (Defranchi et al. 2011; Gross et al. 2007; Johnstone et al. 2010; Noraberg et al. 2005; Schultz et al. 2015; Sundstrom et al. 2005; van Vliet et al. 2007; Wang et al. 2008; Yla-Outinen et al. 2010). Additional proof-of-concept studies on electrophysiological activity have also been completed in three dimensional (3D) re-aggregating rat brain cell cultures (van Vliet et al. 2007). These data suggest the utility of *in vitro* and *ex vivo* neuronal models in identifying the chemical impact on electrophysiological parameters such as neuronal excitability and depression potential.

9.2.3 Multi Electrode Arrays to Model Electrophysiological Changes Upon Drug Treatment

Conventional single well measurements of electrophysiological endpoints have low throughput and are not conducive to higher throughput drug screening. Existing higher throughput models focus on single targets, and utilize artificial systems such as lipid bilayers and recombinant expression in non–neuronal cells (Dale et al. 2007; Le Pioufle et al. 2008). The emergence of multi electrode arrays (MEA) that increase the capacity for higher throughput have opened up the field to screening compounds for effects on neuronal function regardless of the mechanism of action (MOA) (Defranchi et al. 2011; McConnell et al. 2012; Novellino et al. 2011; van Vliet et al. 2007; Yla-Outinen et al. 2010). Additionally, using MEA technology, the functional impact on more than one target can be assessed on neuronal networks by a multitude of compounds with the capacity to disrupt a variety of pathways (Johnstone et al. 2010; McConnell et al. 2012).

These electrophysiological techniques record signals from electrodes present at the bottom of cell culture plates and can include recordings of the number of spikes per channel, mean firing rates and bursts, mean

burst durations in time, and spikes in bursts along with several other parameters (Johnstone et al. 2010). The MEA also enable the extracellular recording of electrically active signals from multiple sites (Johnstone et al. 2010). Cells (or tissue slices) can be seeded as a monolayer on top of electrodes covering the bottom of the cell culture plate to capture electrical activity elicited by action potentials from cells in the vicinity (Johnstone et al. 2010). This technology offers a non-invasive, marker free assessment of functional activity that can be used to take repeated endpoint measurements to assess longitudinal effects. The ability to assess early functional effects is of value, since in many circumstances electrophysiological changes may occur prior to morphological changes (Melani et al. 2005; van Vliet et al. 2007).

However, there are limitations of cell-based electrophysiology models using primary cells including the time it takes for cell preparation, growth, and subculture conditions (generally weeks to months), high animal or embryo numbers needed for each study, and the cost of obtaining primary cells from rodents (or humans) including hippocampal slices and stem cells. These models are also limited to sourcing cells mainly from animals, requiring tissue harvest and therefore donor-to donor variability, as well as skilled dissection and preparation of cultures, which can result in further variation. Despite these limitations, *in vitro* neuronal cultures such as fetal rat cortical neurons have shown reproducibility and relevance in assessing chemical impact on electrophysiological parameters similar to that reported *in vivo* using the same chemicals (Novellino et al. 2011). Furthermore, the effects on electrophysiological endpoints can be seen with compounds that not only have a direct effect on the modulation of ion channels and receptors but also may capture mechanisms derived from metabolic or structural disruptors (Johnstone et al. 2010).

9.3 Cell Types that Can Potentially Be Used for *In Vitro* Neurotoxicity Assessment in Drug Development

9.3.1 Primary Cells Harvested from Neuronal Tissues

Primary cultures derived from nerve tissues have been used to study the nervous system both at the cellular and molecular level. This approach has helped to gain insight into the functional aspects of neurons and supporting cells both in the developing and adult brain. However, human sources of neuronal cells are extremely difficult to obtain as they are generally acquired from aborted human fetuses (including fetal brain tissues or human ESC), patients who have undergone brain surgery, and post mortem human adult tissues (Breier et al. 2010; Sterneckert et al. 2014) (Table 9.1). A major disadvantage of this acquisition process is contamination with either non-neuronal cells or unhealthy cells or a combination of both. Primary rodent tissues such as DRG cultures avoid the ethical and technical concerns associated with obtaining human tissues, and hence have been utilized in neurodevelopmental and chemotherapeutic safety assessments. However, these DRG cultures do not come from the target species (i.e., humans) and hence may not fully represent the key characteristics being investigated for the target population (Melli and Hoke 2009). Model systems such as neurons derived from ESC and fetal preparations (not covered extensively in this chapter) share semblance with cells *in vivo*, and may retain functional aspects specific to neurons such as the capability to form synapses, generate action potentials, and neurites (Table 9.1).

ESCs are isolated from the inner cell mass of blastocyst stage embryos, and neural progenitor cells (NPCs) on the other hand, are multipotent and can be derived from embryonic, fetal, as well as adult tissues. Most of the work with ESC- or NPC-derived neuronal cells has been focused around the fields of neurodegeneration and tissue replacement therapies (Breier et al. 2008; Reubinoff et al. 2001; Richardson et al. 2006; Roy et al. 2000). Primary cultures have also been utilized for DNT hazard identification since many of the key features of a developing nervous system can be captured. This includes stem cell development, progenitor commitment, and differentiation (Bal-Price et al. 2010b). These primary stem and progenitor cell cultures offer a renewable

Table 9.1 *Cell models amendable to pharmaceutical assessments*

Cell type	Usefulness	Limitations	References
Primary neuronal cells from following sources: i. Adult or fetal tissues ii. Embryonic Stem Cells iii. Neural Progenitor Cells	• Both human and rodent sources of origin • Contain cells that can recapitulate cell types from *in vivo* environments either alone or in mixtures • Renewable cell source • Established methods for isolation and propagation	• Increased cost and time for cell preparation, and maintenance • Limited source for human tissues • Ethical concerns arising from human sources • Less amendable to higher throughput screening	(Breier et al. 2010; Breier et al. 2008; Buzanska et al. 2006; Coecke et al. 2006; Coecke et al. 2007; De Filippis and Binda 2012; Harry and Tiffany-Castiglioni 2005; Reubinoff et al. 2001; Robinette et al. 2011)
Immortalized cells	• Both human and rodent sources of origin • Reproducible • Unlimited availability • Large scale production to aid screening • Cost and time effective • Limited ethical concerns	• Tumor derived sources may lack representation of normal molecular underpinnings • Potential for genomic instability	(Breier et al. 2008; Coecke et al. 2007; Donato et al. 2007; Harrill et al. 2011; Harry and Tiffany-Castiglioni 2005; Radio et al. 2008; Xia et al. 2008)
Induced pluripotent stem cells (iPSC) derived neurons	• Both human and rodent sources of origin • Unlimited availability • Reproducible • Large scale production to aid screening • Disease specific neuronal cells • Cost and time effective • Limited ethical concerns	• Somatic derived cell types may lack molecular characteristics of true neurons	(Parent and Anderson 2015; Tada et al. 2001; Takahashi et al. 2007; Takahashi and Yamanaka 2006; Yamanaka 2012; Yu et al. 2007)

source of cells that can be differentiated into any cell belonging to the nervous system. Cells can also be isolated from either sex and from different stages of development. Due to the availability of inbred rodent strains, they also offer some consistency between preparations from different individuals (Breier et al. 2008). The ability to differentiate into multiple neuronal subtypes has made ESC- or NPC-derived neuronal cells attractive in research pertaining to neurological diseases including Parkinson's Disease (PD), Alzheimer's Disease (AD), and Huntington's Disease (HD) (Conti et al. 2005; Donato et al. 2007; Sterneckert et al. 2014). Preparation and maintenance of ESCs or NPCs is a labor intensive and expensive process, and generates a heterogeneous population, both morphologically and functionally. In addition, fetal tissues are generally difficult to obtain in large quantities making these cultures less practical for screening purposes since reproducibility of the results rely on the preparation protocols and post isolation characterization for each batch. However, commercially available sources are emerging that enable the acquisition of primary cell preparations that have undergone basic characterization, thereby facilitating reduction in variability of the data generated (Breier et al. 2010). Cell sources include, but are not exclusive to the National Institutes of Health (NIH) stem cell registry, which currently has a comprehensive list of available cell lines and immortalized

fetal derived cells (Cacci et al. 2007; De Filippis and Binda 2012). Cell culture preparations pose long-term difficulty in maintenance and can exhibit contaminating cell types due to the constant need to prepare new cultures. Additionally, assessment of neurotoxicity on mature cells requires long lead times for differentiation; as a result, the cells may lose genotypic or phenotypic stability over time. Taken together, these very traits described here may prove difficult for high throughput applications in a pharmaceutical industry setting (Breier et al. 2010), but they may still be useful for hypothesis-driven investigational activities related to specific mechanistic studies.

Cord blood stem cells are a human derived NPC source that may show promise for screening purposes. Buzansak et al. used human umbilical cord blood stem cells to generate the neural progenitor line HUCB-NSC (human umbilical cord blood-derived neural stem cell), which was evaluated for its utility to assess DNT (Buzanska et al. 2006). These cells demonstrated expression of pluri- and multi-potent markers as non-differentiated floating stem cell aggregates and could be differentiated into neurons, astrocytes, and oligodendrocytes (Bal-Price et al. 2010b; Buzanska et al. 2006, 2009). These cells also exhibited the expression of functional proteins such as glutamate and GABA (gamma-aminobutyric acid) receptors, voltage and ligand gated ion channels, and functional networks (Jurga et al. 2009; Sun et al. 2005). Importantly, the cells were utilized to assess viability, proliferation, and cell differentiation after exposure to environmental developmental toxicants and demonstrated a greater sensitivity to toxicants in their early stages of development compared to later stages showing proof of concept for screening chemicals for DNT in this model.

9.3.2 Immortalized Cells and Cell Lines

Cell lines are generally clonal in origin and commonly immortalized with an oncogene such as Myc to retain the proliferative potential and characteristics of the original population (Breier et al. 2008; Harry and Tiffany-Castiglioni 2005). Established cell lines have the advantage of providing a constant source of relatively homogenous cells that can be cost effective and easily handled in culture (Table 9.1). These cells can also be generated in large quantities making them more easily amendable to high throughput assay systems. On the other hand, cell lines may have limitations in mimicking the mechanistic underlying causes of toxicity or accurately assessing neuronal subtype effects (Coecke et al. 2007; Deshmukh et al. 2012; Greene and Tischler 1982; Harry and Tiffany-Castiglioni 2005).

Nonetheless, many immortalized cell line models have been utilized to assess both the neurodevelopmental impact of chemicals and chemotherapeutic effects on neurite dynamics. Commonly used models include mouse neuroblastoma cell lines including N2a and NIE-115 (Richelson 1973a,b,), human neuroblastoma cell lines including SK-N-SH and SH-SY5Y, and the rat pheochromocytoma cell line like PC-12 (Arastu-Kapur et al. 2011; Ceresa et al. 2014; Geldof et al. 1998; Nakagawa-Yagi et al. 2001; Scuteri et al. 2006; Takadera et al. 2010; Tomassoni et al. 2013; Verstappen et al. 2004; Wienecke and Bacher 2009). Data generated from these *in vitro* rodent derived cell models require extrapolation to humans. While the SK-N-SH and SH-SY5Y cell lines are species-relevant, they are also cancer-derived, and therefore may not be representative of normal human neurons. Immortalized tumor cells contain tumor growth genes that may affect the result of assessing responses to chemicals for neurotoxicity testing. One example is the overexpression of MEIS homeobox gene in neuroblastoma cells which can influence developmental signaling pathways, proliferation, and apoptosis (Bal-Price et al. 2010a,b; Geerts et al. 2003). The rodent pheochromocytoma PC12 (Green 1977) is a model widely used to assess neurotoxicity effects including neurite changes upon chemical treatment. However, one caveat is that these formed neurites do not exhibit the properties of either axons or dendrites (Banker and Goslin 1998; Greene LA 1977, Tischler and Greene 1978).

Although many of these *in vitro* cell lines do recapitulate the general traits of neurite dynamics upon chemical treatment, human and rodent cells may vary in sensitivity as well as exhibiting opposing or complimentary effects. In addition, normal cells and tumor cells of the same species can differ in their sensitivity to

chemicals as well (Harrill et al. 2011; Radio et al. 2010). Even closely related cell lines such as SK-N-SH and SH-SY5Y, where the former is the parental line of the latter, can show different patterns of compound induced cytotoxicity (Xia et al. 2008). This is exemplified by differential responses from a comprehensive screen measuring cytotoxicity in human SK-N-SH and SH-SY5Y lines after treatment with over 1300 selected compounds based on toxicology data from standard tests (carcinogenicity, genotoxicity, immunotoxicity, reproductive, and developmental toxicity). Nonetheless, tumor cells may still be useful for certain applications and it is essential to have an understanding of the mechanistic underpinnings of the effect studied prior to setting up a model (Bal-Price et al. 2010a,b; Coecke et al. 2007; Kohl et al. 1980, 1983; Reynolds and Perez-Polo 1975; Wilson et al. 2014).

Human immortalized fetal NPC are now commercially available (ReNcell, Millipore) and have been utilized in a screening assay for neurotoxicity effects. The ReNcell cortex (CX) and midbrain (VM) NPC were originally isolated from human fetal tissues and immortalized using the Myc oncogene (Donato et al. 2007). This may limit their utility in assessing effects on neural cell proliferation (Bal-Price et al. 2010a,b; Breier et al. 2008), but several other aspects make them a potential source for screening. The cell lines display a normal karyotype, express immature markers (e.g., Nestin and sox2), and can generate neurons, astrocytes, and oligodendrocytes upon mitogen withdrawal. The VM cell line specifically has the capacity to generate action potentials (Breier et al. 2010a,b; Donato et al. 2007). This latter cell line was utilized to develop an automated HTS method that was validated with a set of chemicals known to cause developmental toxicity. Chemical effects on cell proliferation (BRDU assay) and viability (involving propidium iodide) (Bal-Price et al. 2008) in ReNcell were evaluated in a HTS manner and the obtained data highlighted the potential utility of adding human NPC (hNPC) in the battery of tests to screen chemicals for neurotoxicity effects. This data may indicate the utility in additional validations to test functional aspects of compound effects on conductivity and synapse formation.

9.3.3 Induced Pluripotent Stem (iPS) Derived Cells

iPSCs are derived from adult somatic cells by genetic reprogramming (Takahashi and Yamanaka 2006). Similar to ESCs, they are capable of self-renewal, are pluripotent, and can form all cell types except extra-embryonic tissues (Tada et al. 2001; Takahashi et al. 2007; Takahashi and Yamanaka 2006; Yu et al. 2007). Somatic cells are generally reprogrammed via virus–based systems, episomal systems, or mRNA/miRNA-based systems, with the former two avoiding issues with mutagenic events due to genomic integration (Robinton and Daley 2012; Yamanaka 2012).

The methods for neuronal generation using iPSCs are still in progress and the protocols for generating neurons between different research laboratories have not yet been standardized. Neuronal-derived cells from different sources differ in their maturity, quality, and purity with potentially unknown differences at the molecular level. As with the generation of ESCs, there may be variability in DNA methylation, transcriptional, and epigenetic factors between various iPSC preparations and general variability between preparations (Bock et al. 2011; Boulting et al. 2011; Dolmetsch and Geschwind 2011; Marchetto et al. 2010; Robinton and Daley 2012). iPSC-derived neurons are generated in academic laboratories for basic research applications and mechanistic studies with particular emphasis in the neurodegenerative field (Burkhardt et al. 2013; Dolmetsch and Geschwind 2011; Juopperi et al. 2011; Marchetto et al. 2010; Nguyen et al. 2011; Odawara et al. 2014; Parent and Anderson 2015; Park et al. 2008; Urbach et al. 2010).

Human iPSC-derived neurons are also commercially available from a number of suppliers. They are described as being differentiated into matured neurons from reprogrammed pluripotent cells obtained from both healthy and diseased human donors. Human iPSC-derived neurons from commercial sources have facilitated the development of neurotoxicity assays in an industrial setting. The use of human iPSC-derived neurons can overcome limitations of current *in vitro* cellular models by providing an unlimited supply of pure

human specific cells from a standardized method. iPSC-derived cells circumvent species extrapolation, avoid ethical concerns, and provide a consistent reproducible phenotype. Such advantages enable the development of higher throughput applications and lower both inter and intra assay variability.

Another major advantage for using iPSC-derived neurons is that vendors provide characterized matured phenotypes ready for application, circumventing the need to mature and differentiate cells for long periods of time. In addition, several cell types such as neurons and astrocytes can be generated from the same genetic background, thereby providing the possibility of studying toxicity in the context of human genetics. The homogenous nature of the preparations, the ease of handling, and propagation for long periods of time lends itself to investigating both acute and long term toxicity effects of drugs on neuronal function and development. In addition, in contrast to ES cells, iPSC-derived neuronal cell types can be generated from normal human phenotypes as well as patient populations with neurodegenerative diseases, neuropsychiatric disorders, and individuals with specific mutations. Owing to the plethora of sources for its generation, the iPSCs enable the study of mechanism of pathology as well as the effect of genetics on disease penetrance (Burkhardt et al. 2013; Dolmetsch and Geschwind 2011; Juopperi et al. 2011; Marchetto et al. 2010; Nguyen et al. 2011; Okano and Yamanaka 2014; Parent and Anderson 2015; Park et al. 2008; Pasca et al. 2011; Prilutsky et al. 2014; Sanchez-Danes et al. 2012; Urbach et al. 2010). These cells are particularly interesting in the context of neurodegenerative disease research, where animal models have limited translatability and a previous lack of human neuronal cultures that can mimic the disease pathology (Khurana et al. 2015). The challenge is to demonstrate that the phenotypes of the resulting iPSC-derived neurons are relevant to patients. Several companies such as Bristol-Myers-Squibb, GlaxoSmithKline, and Roche have utilized patient derived iPSCs to aid in the development of clinical candidates for neurodegenerative diseases (Mullard 2015). These patient-derived neuronal models of AD, ALS (amyotrophic lateral sclerosis), PD, and HD have been used for target identification, lead validation and optimization, efficacy, and pharmacological screening assessments to select candidates. While iPSC-derived cell models are now being used as a tool to aid in clinical drug candidate selection, the emerging information from clinical trials may help to clarify the correlation of iPSC-derived phenotypes and the relevance to patients (Mullard 2015).

Some vendors that provide human iPSC derived neuronal cell types include; Reprocell, Aruna Bioscience, Axol, Globalstem, Axiogenesis, and Cellular Dynamics. Several of these companies such as Axiogenesis, Aruna Bioscience, and CDI (Cellular Dynamics Inc.) sell products for human iPSC-derived neuronal subtypes (DA, motor neurons, peripheral neurons), astrocytes, and kits for differentiation of neural progenitor and stem cells and region-specific (cortical–Axol) neurons. Commercially available iPSC neurons have also been shown to display a typical neuronal morphology with the formation of neurite networks, exhibiting the expression of mature neuronal markers such as MAP2, Tuj1, BIII tubulin, and synaptic markers such as synapsin, Vglut, and Vgat. As with other cell types generated from iPSC-derived protocols, the neuronal cultures have been shown to display immature features (including expression of Nestin and double cortin, DCX) as well as mature features described herein. Our laboratory has characterized commercially available human iPSC-neurons both transcriptionally and at the protein level (unpublished data). The majority of the cells tested exhibited a stable expression profile over time in culture and expressed a wide variety of neuronal specific markers, but simultaneously exhibited immature features comparable to fetal tissues.

iPSC-derived mature cell types are currently integrated in the drug development process in safety assessment approaches (Anson et al. 2011). An example is the use of the iPSC-derived cardiomyocytes, which are integrated in the safety assessment process in early drug discovery, and is showing great promise in identifying certain molecules with reference to cardiac liabilities (Guo et al. 2011; Harris et al. 2013; Kolaja 2014; Ma et al. 2011; Peng et al. 2010). The use of human iPSC-derived neurons for neurotoxicity testing is an emerging field in risk assessment for drug development. Advanced research and direction is needed around characterization and validation of cells and models. Such insights will aid significant development of the field and help in developing testing as a part of routine screening. These cells, as with any *in vitro* assay, need to

be evaluated in the context of the pharmacological effect it is expected to model as well as capture. Initial progress suggests that these cells may show promise for human risk assessment in a pre-clinical setting. The next section will detail some examples of the uses of iPSC in safety assessments particularly pertaining to the detection of functional changes such as effects on neurite dynamics and electrophysiology.

9.4 Utility of iPSC Derived Neurons in *In Vitro* Safety Assessment

9.4.1 iPSC Derived Neurons in Electrophysiology

Early studies with iPSC-derived neurons show that they can exhibit spontaneous and evoked action potentials in culture as well as display compound induced changes in network activity (different vendor posters: Glenn et al. 2014; Ott et al. 2013, Pasca et al. 201; Woodard et al. 2014). Initial studies have shown the presence of Na, K, and Ca-channels in iPSC derived neurons (Haythornthwaite et al. 2012). One noticeable limitation was the time window used to capture robust spontaneous action potentials *in vitro*. This was improved in co-cultures with astrocytes demonstrating prolonged culture time with the capacity for spontaneous firing (Odawara et al. 2014). Modulation of spontaneous action potentials with inhibitory compounds such as eserine or nifedipine re-capitulated inhibitory effects that were reversible upon withdrawal of compounds (unpublished data). However, modulation of the spontaneous action potentials when exposed to excitatory compounds did not readily recapitulate excitatory responses in these neurons. This is likely the result of these neurons exhibiting a glutamatergic or GABAergic phenotype, which is predominantly inhibitory in nature. However, the emergence of iPSC-derived subtype-specific neurons would make it possible to create a mixture of excitatory and inhibitory neurons to test compound effects for future applications.

9.4.2 iPSC Derived Neurons to Study Neurite Dynamics

High content and higher throughput screening models for neuronal toxicity utilizing iPSC-derived neurons and astrocytes have been developed by several companies and investigators (Efthymiou et al. 2014; Sirenko et al. 2014; Snyder et al. 2014, 2015; Wheeler et al. 2015). In a proof-of-concept study, Sirenko et al. demonstrated the use of human iPSC-derived neurons in high throughput high content screening systems (Sirenko et al. 2014). Human iPSC-derived neurons were cultured in a 384-well format using Calcein AM (for fluorometric determination of live cells) and Hoechst anti-β-III tubulin to assess the effects of compounds on cell viability, neurite outgrowth, and morphological parameters. These initial attempts show promise in using iPSC-derived neurons to screen compounds for neurotoxic effects in a miniaturized higher throughput assay format. Some limited validation studies have also demonstrated the potential of these cells to capture toxicities seen in the clinic with chemotherapeutics. Initial studies testing a limited set of chemotherapeutic compounds with known neuropathic potential on human iPSC-derived neurons show promise in the detection of neurite dynamic changes and viability (Snyder et al. 2015; Wheeler et al. 2015). Endpoints evaluated included Caspase3/7 to evaluate apoptosis, Hoechst for nuclear identification, and Calcein AM to visualize the cytoplasm and effects on neurites using high content imaging systems (Wheeler et al. 2015). Dose dependent changes in these parameters enabled the identification of compounds with a higher or lower induction of toxicity. Similar to these initial studies, we have developed a live cell kinetic assay utilizing human iPSC-derived neuronal cells to assess the pathophysiological impact of a larger initial test set of chemotherapeutics that result in clinically dose-limiting neuropathy (Fig. 9.3/Plate 4). Live cell imaging has the advantage of being able to capture long-term, longitudinal effects of drug treatment on neuronal function in a non-disruptive manner. Accumulative effects, threshold effects, maturation or developmental stage effects and the reversibility of these effects with wash out periods is conceivable with these approaches since information

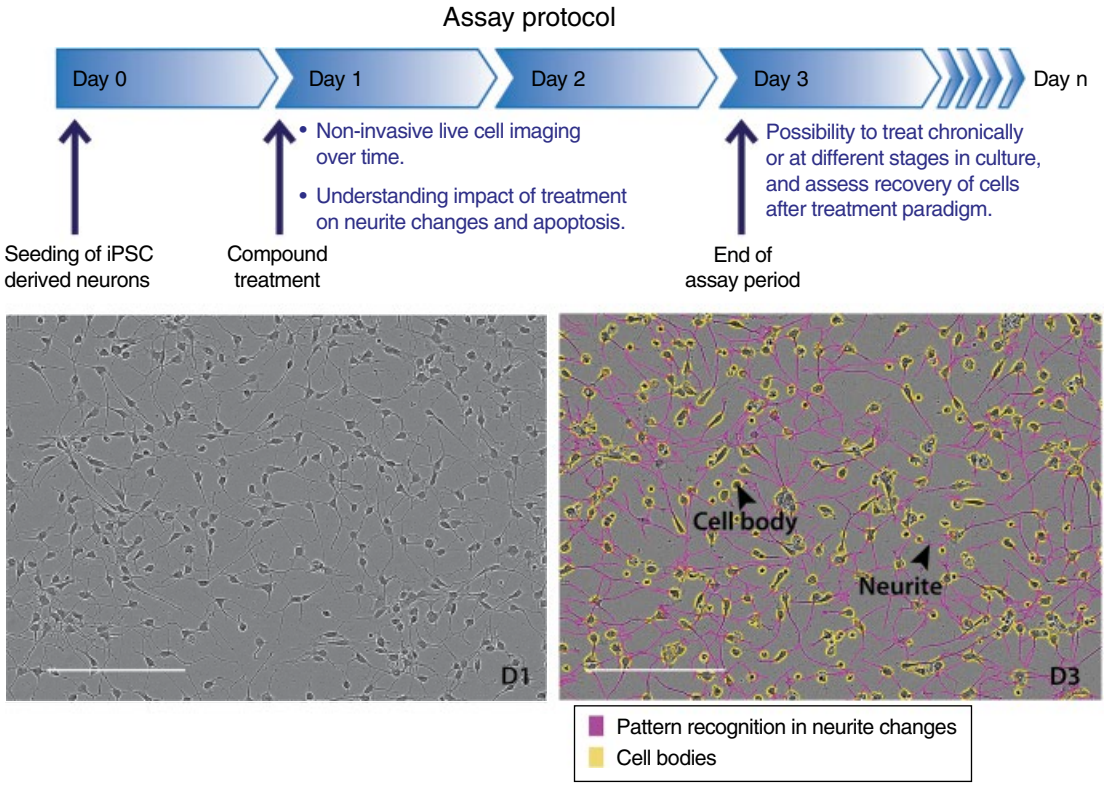

Figure 9.3 (Plate 4) *Flexible dosing and scanning scheme for non-invasive live cell imaging over time using iPSC derived neurons. Cell cultures are maintained in a CO_2 and temperature controlled setting. Changes in neurite dynamics are observed during the experimental timeline, including apoptosis by Caspase 3/7 measurement (not shown). Pattern recognition software enables analysis of neurite length and cell bodies within each well measured over a period of time. D1 indicates Day 1 when the cells are treated and D3 represents Day 3 as the end of assay period. Scale for images is 200 μm. (See insert for color representation of the figure.)*

from the same well location of neurons is captured over time. Development of a kinetic, high-resolution imaging model has allowed multiplexing of endpoints for neurotoxicity such as assessing neurite dynamics by phase contrast microscopy and evaluating apoptosis via fluorescent measurement. In this model the pattern recognition software enables reliable identification and quantification of neurite length and cell bodies in a homogenous cell population measured over time (Figs 9.3, 9.4/Plates 4 and 5). In parallel, the green fluorescence channel, along with a green fluorescent dye specific for active Caspase 3/7, measures the number of iPSC-neurons undergoing apoptosis. Cumulative data obtained from this model provides significant insight into the effect of the compound being tested on neuronal function. The validation set of compounds represented in this model includes multiple classes of chemotherapeutics, many of which cause dose-limiting neuropathy in clinical trials such as microtubule stabilizers, microtubule de-stabilizers, DNA cross-linkers, and proteasome inhibitors. Chemotherapeutic dose-dependent changes in neurite dynamics and apoptosis parameters can be detected in this model. Rank order information aids in identifying the microtubule effector vincristine as the most toxic of the three chemotherapeutics tested followed by paclitaxel and cisplatin, in line with clinical findings and results with primary *ex vivo* neuronal models and iPSC-derived neuronal work performed by other groups (Malgrange et al. 1994; Wheeler et al. 2015; Wilson et al. 2014).

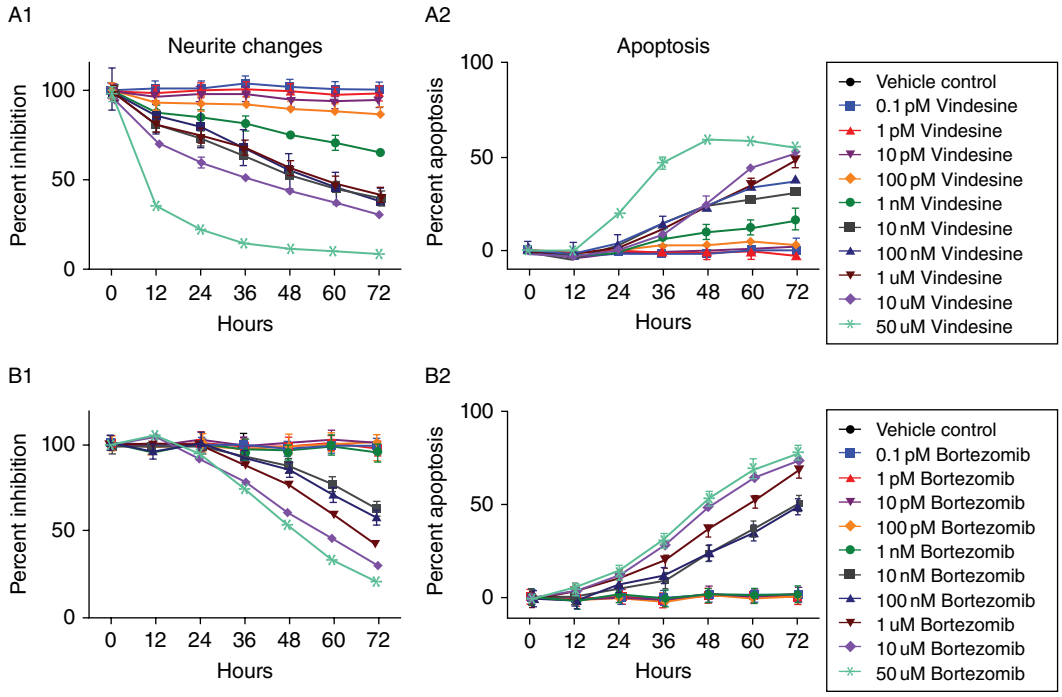

Figure 9.4 ***(Plate 5)*** *Differences in neurite changes and apoptosis upon treatment with varying doses of microtubule de-stabilizers or proteasome inhibitors on iPSC derived neurons over time. Live cell imaging over time and the capability to incorporate multi-parametric approaches in this assay enables the identification of molecules with different mechanism of action and may increase the sensitivity and neuro-specificity of the assay. Panel A. Microtubule de-stabilizer Vindesine induces neurite changes (A.i) prior to detection of apoptosis (A.ii). Panel B. Proteasome inhibitor Bortezomib induces concomitant toxicity in both parameters (B.i and B.ii). (See insert for color representation of the figure.)*

Multiplexing endpoints additionally enabled the classification of tested compounds into two defined classes based on the sensitivity of the two endpoints to increasing concentrations of the test compound over time (Fig. 9.4/Plate 5). These data highlight the importance of including multiparametric approaches incorporating pharmacology/MOA and phenotypic outcome to increase sensitivity or potential predictive value (Wheeler et al. 2015). An integrated approach of these types of functional assays with multiple endpoints provides sensitive data that could enable classification based on MOA and rank ordering of compounds to inform decision-making during the drug development process. Additional endpoints such as respiration, oxidative stress, mitochondrial health, and transcriptional profiling can be added as needed for mechanistic studies.

One drawback with these models is the reliance on CNS defined iPSC-derived neurons and the limited availability of human peripheral iPSC-derived neurons. The emergence of iPSC-derived peripheral neurons (PN) will enable comparison of results in both systems. Nevertheless, initial studies indicate that the rank order of toxicity for a limited test set of chemotherapeutics show similar results when comparing data generated from rodent DRG or a peripheral iPSC-derived cell source (unpublished data) (Malgrange et al. 1994; Wheeler et al 2015). While iPSC neuronal sources likely hold relevance for assessing PN effects, they are limited in that they are artificial systems. Continued evaluation will determine how comparable iPSC-derived neurons and central neurons are compared to primary neurons and peripheral cell sources in this assay. The utility of these assays are dependent upon the MOA of the compounds being assessed, and thorough assay validation is critical prior

to drawing conclusions. These studies demonstrate that iPSC-derived neurons can mimic clinical neuropathy effects of chemotherapeutics by inducing neurite changes *in vitro* in response to drug treatment. With the emergence of iPSC-derived neuronal cell subtypes and supporting neuronal cells such as astrocytes, more complex neuronal model systems comprising of mixed populations of human cells can also be developed. Despite the current lack of extensive validation, the human iPSC-derived neurons show considerable promise for use in high-content, high-throughput, and toxicity screening paradigms during safety evaluations of compounds.

9.5 Summary of Key Points for Consideration in Neurotoxicity Assay Development

Current *in vitro* models for neurotoxicity testing are limited in their suitability for accurate prediction and are focused more on the characterization of toxicity (Johnstone et al. 2010). Primary cell models are time consuming, can demonstrate high variability, and show limitations in throughput and scalability making this platform less practical in a drug development setting. Immortalized cells, while not limited by scale or throughput, may have limitations in predicting the mechanism of neurotoxicity since they may lack the exact phenotype of a neuron. Technological advancements have made it possible to query neurotoxicity questions in a pharmaceutical industry setting with higher throughput but to date they mainly rely on rodent primary cultures (Al-Ali et al. 2013; Breier et al. 2008; Gotte et al. 2010; Harrill et al. 2011).

In addition, *in vitro* models are often comprised of less complex systems of single cell cultures that limit the recapitulation of complex, 3D neural circuitry, and the involvement of supporting cells. The PNS and CNS consist of highly complex cell networks, which show regional specificity. Once isolated, these cells lose their integrated function and may undergo phenotypic and genotypic changes in culture that may not reflect their true function *in vivo*. The importance of supporting cells in neurotoxicity assessments has been shown for environmental chemicals (Coecke et al. 2006; Dragunow 2008; Lobsiger and Cleveland 2007; Mika et al. 2013; Miller et al. 2004; Sisignano et al. 2014), where part of the pathology or MOA of toxicity involves supporting cells and immune cells. More complex assays that investigate the effects on supporting cells (e.g., glial, oligodendrocytes, microglia, and Schwann cells), the impact of immune system components, cellular metabolism, and BBB function are emerging (Breier et al. 2010; Reynolds and Rietze 2005). Models that utilize whole brain aggregates retain some of these functional aspects but are limited to animal species that may not reflect the genetic or phenotypic composition of the human system. The emergence of more complex 2D or 3D cultures exemplified by neurosphere cell cultures, BBB models, mixed neuronal cell cultures (including supporting cell types and immune components), and organotypic cultures may enable investigations in a more integrated manner (Breier et al. 2010; Lancaster et al. 2014; Naik and Cucullo 2012; Reynolds and Rietze 2005). Due to the complex nature of the CNS and PNS, no single *in vitro* system will likely be relied upon for toxicity assessment. Therefore, multiple endpoints and systems may be evaluated followed by interpreting the data in an integrated manner. The utility of *in vitro* assays to study and assess neurotoxicity will depend on the choice and validation of appropriate models that exhibit characteristic molecular and cell signaling mechanisms with the ultimate goal of allowing for quantitative and reproducible measurement of endpoints at a good dynamic range.

Additionally, with the development of more sophisticated and commercially available technical advances such as multi-electrode array recordings, microfluidics systems, and the commercial availability of both primary and iPSC derived neuronal cells, it is possible to query the function of neurons on a higher-throughput and long-term basis (Johnstone et al. 2010; Yang et al. 2009). In addition, iPSC-derived neuronal cells from disease patients can potentially be used to capture genetic variations and drug susceptibility, while also providing a tool for testing the influence of genetic and environmental factors on neurotoxicity.

These novel approaches and the emergence of commercial cell sources offer increased opportunities to explore alternatives in an integrated manner in both rodents and the target species (i.e., humans). Development

of assays for mechanistic studies, screening, and rank ordering of compounds based on toxicity requires an understanding of the chemical impact on cellular functions to be measured; most often this understanding is still incomplete. A test set of compounds is typically utilized when validating or qualifying cell models and assessing the utility of these models as screening tools for hazard identification. Data generated from a test set of compounds with known clinical neurotoxicity and compounds that lack clinical neurotoxicity may help inform the potential clinical translatability of *in vitro* effects and assess the predictive value of the assays. The examples presented come from the field of developmental neurotoxicity and the pharmaceutical industry where test sets and higher throughput attempts have been undertaken (Breier et al. 2010; Carrier et al. 2006; Defranchi et al. 2011; Gartlon et al. 2006; Johnstone et al. 2010; Novellino et al. 2011; Radio et al. 2010) with toxicants and chemotherapeutics, and such relationships are being assessed (Arastu-Kapur et al. 2011; Defranchi et al. 2011; Johnstone et al. 2010; Luber-Narod et al. 2001; Wheeler et al. 2015; Wilson et al. 2014).

For the purpose of utilizing an assay for hazard identification in drug development and in an industrial setting several features may be advantageous. These features are described as follows:

1. Constant, reproducible source, and adequate volume of cells for data reproducibility. This will enable HTS with limited variability, which is otherwise introduced either alone or as a cumulative effect by using different methods of isolation, propagation, and differentiation.
2. Time and cost effectiveness in terms of handling and propagation. This would limit the efforts to harvest and isolate primary tissues for prolonged cell periods, and reduce animal use, which in turn enables faster turnover of data to help make decisions around a molecule (using hazard identification and MOA).
3. Characterization and validation of cellular processes and endpoints of interest. Validation of the assays to include assessing test sets of chemicals (retrospectively or prospectively) with known mechanisms of toxicity to measure either general cell health parameters, neurospecific endpoints, or a combination of both. This process enables confidence in the assay's predictability for accurate hazard identification.
4. Cross-laboratory reproducibility to help ensure that the results are repeatable.
5. Choice of relevant species. Clinical de-risking would entail testing in human models or in alternative models from other species where the molecular underpinnings mimic the human mechanism of action. Establishing translatability from pre-clinical to clinical findings is an elaborate process, which may require insight into the basic biology or data from distinct historical data sets for compounds with known clinical neurotoxicity. There is a need to establish translatability of *in vitro* models to pre-clinical and clinical findings. Such a model needs to be validated for the endpoint measure as characterized by adequate mimicking of the specific molecular process of neurotoxicity (Breier et al. 2008; Radio et al. 2008).
6. Adequate endpoints. Historically, neuronal specific endpoints have not been routinely added to *in vitro* tests. Further, the general cell health endpoints were relied upon for assessing the neuronal specific toxicity of compounds. To increase the confidence in the specificity of the tested compound, a combination of both functional neuronal effects as well as cell health effects could be utilized.
7. Multiplexing different endpoints and the inclusion of non-neuronal cell types for potency comparisons enables qualification of cell specific toxicity and sensitivity. The collection of a multitude of information to capture the effects of chemical impact on cell function may aide in identifying the mechanism of toxicity. Additionally, cell specific endpoints compared to general cell health endpoints can identify neuronal specific functional effects uncoupled from cytotoxicity. Specifically, cross validation with a non-target cell type can be done to compare cell specificity of the tested compound with its effects and sensitivity spectrum across various cell types.

The accumulation of all this information will help to address whether certain cell types are more sensitive than others with respect to hazard identification.

Despite the shortcomings in the current testing paradigms, events that mimic some cellular and molecular pathways involved in cell maturation, commitment, neuronal function, and supporting cell interactions can be captured in *in vitro* systems as mentioned in previous sections. In addition, novel neuronal cell models are emerging that may provide added value in a higher throughput and reproducible fashion. Emerging technologies and models could be integrated into drug development to provide information around hazard identification, dose response assessment, and risk characterization (Harry et al. 1998).

9.6 Concluding Remarks

The application of *in vitro* cell models for neurotoxicity testing is an emerging field within drug development. Technical advancements, as well as the emergence of more characterized and complex cell model systems show promise in addressing specific neurotoxicity questions in a higher throughput and more physiologically-relevant manner. Several investigators have demonstrated the potential utility of incorporating neuronal specific models into drug development for target identification and validation. Additional incorporation of *in vitro* screening- and mechanistic-tools such as electrophysiological endpoints or the investigation of compound-related changes in neurite dynamics could provide added value together with animal studies in hazard identification and risk assessment, with the goal to reduce the clinical attrition based on neurological findings. As with any *in vitro* approach it is important to validate and address potential translation of findings and molecular underpinnings of the effect to be investigated. Based on the often limited understanding of underlying MOA of toxicity, caution should be taken in the interpretation of data since predictivity and translation to human from *in vitro* models are not readily established. Instead, a holistic, weight-of-evidence view that incorporates several data points (both pharmacology and *in vitro* information) can help drug development teams make informed decisions around neurotoxicity risk potential and compound prioritization. As the model systems and assays become better characterized and validated, their impact on the drug development process is expected to increase in the context of risk assessment strategies. Ultimately the incorporation of *in vitro* tools into the risk assessment process aides the improvement of chemical design and can help select favorable molecules with less neurotoxic properties and eliminate high risk compounds moving forward. Neuronal specific assays could also identify toxicity associated with new targets and help assess the molecular mechanism of toxicity to potentially risk mitigate and inform clinical monitoring plans with the overarching goal of decreasing the clinical attrition related to neurological findings.

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10

A Human Stem Cell Model for Creating Placental Syncytiotrophoblast, the Major Cellular Barrier that Limits Fetal Exposure to Xenobiotics

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10.1 Introduction

Stem cells, as the collection of papers in this book makes plain, are playing increasingly important roles in toxicological research (Davila, Cezar et al. 2004; Zdravkovic, Genbacev et al. 2008; Kang and Trosko 2011). Such cells can be of particular value as alternatives to the use of live animals, immortalized cell lines, or short-term primary cultures in generating functional cell types whose responses to various kinds of foreign chemicals can then be tested *in vitro* (Rolletschek, Blyszczuk et al. 2004; Sengupta, Johnson et al. 2014; Sjogren, Liljevald et al. 2014). This chapter is explicitly written to explore the possibility of generating placental trophoblast from human pluripotent stem cells and using such cells to test their interactions with xenobiotics and other potentially toxic or teratogenic agents. To our knowledge, there is no precedent for employing such a model system in toxicology, but the authors believe that, if properly applied, it could have utility in examining how compounds with potential to harm the fetus might misdirect placental development and place a pregnancy at risk. The trophoblast cells generated from pluripotent stem cells might also be used to study the transport and metabolism of xenobiotics and their effects on gene networks, especially on the expression of genes encoding detoxifying proteins and transporters. First, however, we provide a brief description of the structure and function of the mammalian placenta, and specifically that of the human. We then indicate why the stem cell-derived model might have advantages over other systems as a

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bio-monitoring tool. Finally, we describe the model itself and provide some unpublished experiments on the changing expression of some genes encoding proteins implicated in metabolism of xenobiotics that accompany the differentiation of human embryonic stem cells into syncytial trophoblast cells.

10.2 General Features of Placental Structure

A placenta provides the interface between the separate blood circulations of the mother and the conceptus (the fetus and its membranes). It is formed by the close apposition or actual fusion of the extra-embryonic membranes with the uterine mucosa, thereby promoting physiological exchange of materials and permitting biochemical information to be passed between mother and fetus. The placenta is not a graft, however. The two blood supplies are not directly interconnected, although the placental surface is in a sense "foreign," as it displays products of paternal as well as maternal genes, and, hence, is at risk of rejection by the maternal immune system. Indeed, perturbations to the delicate immune balance between mother and fetal trophoblast caused by xenobiotics and other foreign chemicals might be one cause of pregnancy loss.

In mammals, where the placenta has reached its highest level of complexity, the basic structure is that of an inner vascular network embedded in connective tissue, connected to the fetus proper by the umbilical veins and arteries, and overlaid by a complex epithelium called trophoblast, which is the region of immediate contact with maternal tissue (Cross 2000). Despite this fundamental organization, which is common to all eutherian mammals, the range of placental structures and the extent to which the trophoblast invades into the uterine endometrium in mammals is bewilderingly complex and a subject beyond the scope of this chapter. More importantly, rodent animal models that precisely mirror the placentation of the human simply do not exist (Carter, Enders et al. 2006).

Despite the range of structures even within single taxonomic groups, most functions, including transfer of nutrients and oxygen from the mother to the fetus and removal of waste products from the fetal blood circulation and returning them to the mother for disposal, are retained across mammals. As a consequence, the placenta is a structure that encounters xenobiotics, including drugs and environmental chemicals, and other potentially toxic and teratogenic agents circulating in the maternal bloodstream (Syme, Paxton et al. 2004; Myllynen, Pasanen et al. 2005; Myllynen, Immonen et al. 2009; Prouillac and Lecoeur 2010). Thus the placenta has a role in buffering the fetus from the effects of these compounds. However, it does not provide an impenetrable barrier to the movement of potential harmful materials into the fetal circulation, but, like the liver, it possesses an active system of enzymes that is capable of detoxifying and sometimes activating harmful compounds and hence contributing to both amelioration and occasional potentiation of toxicity.

10.3 The Human Placenta

The human placenta is flat and roundish, and fused to the uterine mucosa via its basal surface, the basal plate (Benirschke, Kaufmann et al. 2006). It is said to be of the hemochorial type because trophoblast projections, called villi, covered by an epithelium of multinucleated cells, called a syncytium, make direct contact with maternal blood (Fig. 10.1/Plate 6), a situation quite different from that occurring in many other species, especially in ungulates and some strepsirrhine monkeys, where the trophoblast is much less invasive (Carter, Enders et al. 2006). Trophoblast has its origins in the first cell fate decision of the mammalian embryo (McLaren 1982), when the outer cells of the morula preferentially diverge to form trophectoderm, a simple epithelium that constitutes the outer cell layer of the blastocyst, while the innermost cells form the inner cell mass, from which will segregate the extra-embryonic endoderm and the epiblast (Fig. 10.1/Plate 6). The latter

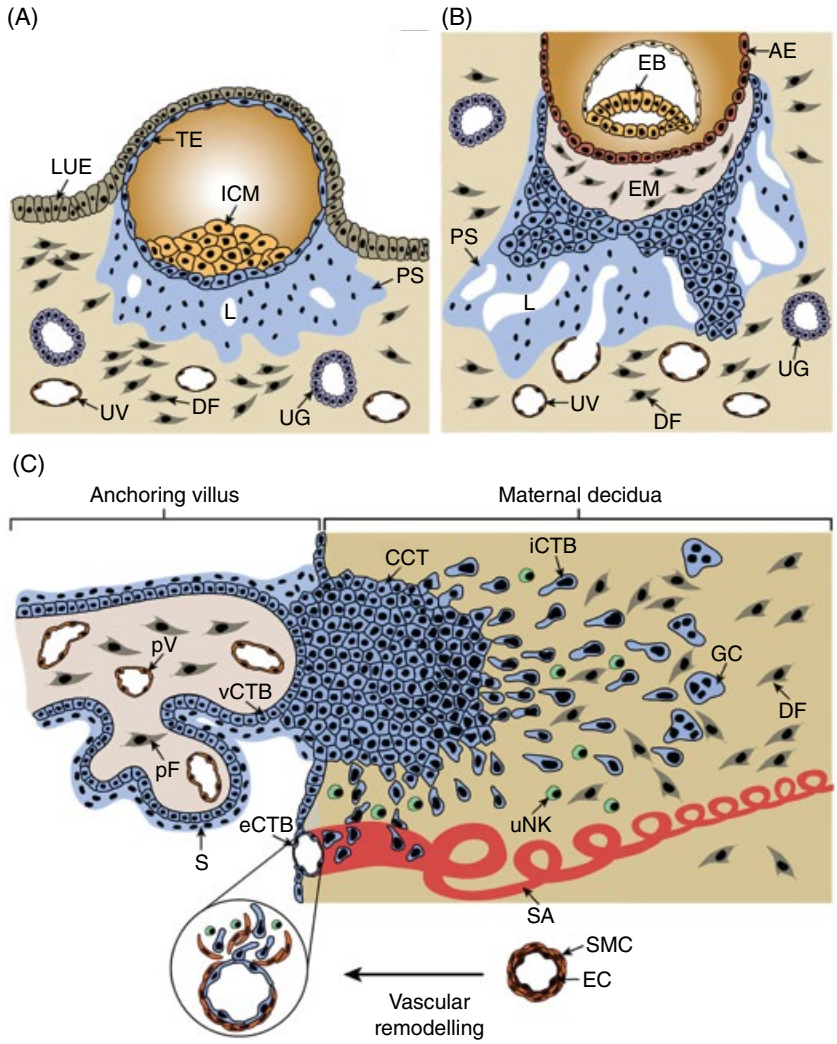

Figure 10.1 ***(Plate 6)*** *Cartoon illustrating some stages in human placental development (Knofler and Pollheimer 2013). (A) After implantation, the conceptus has sunk below the luminal epithelial cells (LUE) of the uterine wall, and proliferating cells of polar trophectoderm (TE) give rise to a primitive syncytium (PS) by cell fusion. This cellular structure appears to be able to invade into the decidualized endometrium and interact with decidual fibroblasts (DF). Non-cellular areas, called lacunae (L) soon inter-connect with uterine vessels (UV), fill with blood, and are the precursors of the intervillous space. (B) Soon after implantation, columns of proliferating cytotrophoblast (CTB) grow through the syncytium to form primary villi. AE, amnion; UG, uterine gland. (C) The architecture of placental villi and the maternal-fetal interface of the human placenta towards the end of the first trimester of pregnancy. Two kinds of villi are encountered: floating villi unattached to maternal endometrium (not shown) and an anchoring villus (shown) that attaches the fetal placenta to the uterine wall. All the villi are covered with a thin layer of STB (S) above villous cytotrophoblast (vCTB) that provides the exchange surface of the placenta. Note that the STB (S) is directly exposed to maternal blood at its apical surface. The core of the villus is comprised mainly of placental connective tissue (pF) and blood vessels (pV). Extravillous trophoblast forms as columns (CCT) at the tips of the anchoring villi and invade into the maternal decidual tissue (DF). A subpopulation of extravillous trophoblast (eCTB) penetrates maternal spiral arteries (SA) and replaces the resident smooth muscle cells (SMC) and endothelium (EC). Other interstitial types (iCTB) penetrate more deeply into the endometrium and encounter maternal NK cell (uNK). So-called giant cells, which are areas of syncytium, are also present in the endometrium, but their origin is not clear. They may arise through fusion of extravillous trophoblast or be remnants of primitive syncytium from the early invasion stages (James, et al. 2012; Knofler and Pollheimer 2013). (See insert for color representation of the figure.)*

is the forerunner of the fetus proper and gives rise to the three germ layers, endoderm, mesoderm and ectoderm beginning at primitive streak formation (gastrulation).

Although the events of early pregnancy in the human are poorly described, it is believed that polar trophectoderm adjoining the inner cell mass makes contact with the uterine wall, begins to multiply to form primitive cytotrophoblast, and breaches the uterine epithelium to which the blastocysts are attached (Fig. 10.1A). A multinucleated syncytium then forms, presumably by cell fusion, ahead of the proliferating, cytotrophoblast cells (Boyd 1970; James, et al. 2012). This syncytium expands into the decidualized stromal layer beneath and appears to be responsible for hollowing out regions within the stroma to form lacunae (Hertig, Rock et al. 1956), which become filled with maternal blood and presumably provide an early source of nutrients for the conceptus (Fig. 10.1B). This primitive placenta will also be exposed to xenobiotics and other foreign compounds at a time in pregnancy when gastrulation is being initiated and a time when many pregnancies appear to fail. By about 12 days of gestation, it is fully encompassed by an outer layer of syncytiotrophoblast (STB) embedded in maternal stromal tissue, strands of cytotrophoblast penetrate through the syncytium to form primary chorionic villi, which, over time, are invaded by extra-embryonic mesoderm and eventually fetal blood vessels to form secondary and tertiary villi (villous trees) (Gude, Roberts et al. 2004; Huppertz 2008; James, et al. 2012; Knofler and Pollheimer 2013), greatly expanding the surface area of the placenta that is available for transport and metabolism (Fig. 10.1C).

Even after the placental villi form (Fig. 10.1C), the cytotrophoblast cells associated with the villi continue to divide and provide a progenitor population, presumably containing trophoblast stem cells, for the overlying villous STB, which is the cell layer that covers most of the outer surface of the villous tree and forms the definitive interface involved in exchange of gases, nutrients, and excretory materials between the fetal placenta and maternal blood (Benirschke, Kaufmann et al. 2006). Villous STB is also the major site for production of placental hormones, such as human chorionic gonadotropin (hCG), placental lactogen (Gaspard, Hustin et al. 1980), and progesterone (Bonenfant, Provost et al. 2000). It is a tissue that turns over rapidly, depositing debris into the maternal bloodstream while being continuously replaced by fusion of cytotrophoblast progenitors from below. Villous STB is also the cell layer of the human placenta that must mitigate against the effects of exposures to a range of compounds, including environmental chemicals such as medications, nicotine, alcohol, and recreational drugs, that pose a threat to the well-being of the developing fetus. As human placenta is readily available at term and sometimes at much earlier time points from pregnancy terminations, there is an extensive literature describing the metabolism of foreign chemicals by the human placenta and also the location of some of the relevant enzymes and transporters, as well as their transcripts within this organ. There is, in addition, a number of papers that describe the presence and concentrations of foreign chemicals, especially heavy metals, in placental tissues and the effect that such chemicals have on the metabolism of the placenta and particularly on its ability to produce hormones, such as hCG. As space to describe these findings is limited, the reader is referred to a number of excellent reviews that cover these topics (Syme, Paxton et al. 2004; Myllynen, Pasanen et al. 2005; Myllynen, Immonen et al. 2009; Prouillac and Lecoeur 2010).

10.4 Human Placental Cells in Toxicology Research

Several *in vitro* methods for studying interactions of foreign chemicals with placenta cells have been described. One approach, for example, has been to perfuse newly-delivered placentas with buffer containing the compound of interest to study transfer from the maternal to the fetal circulation (reviewed by (Myllynen and Vahakangas 2013). Another has been to employ cells of placental origin, including primary cell lines, a number of choriocarcinoma cell lines, such as BeWo, JEG3, and JAR, and lines that have been immortalized from primary cells by genetic manipulation as models for human toxicological research (Prouillac and Lecoeur 2010). Additionally, immortalized rat STB cell lines have been used to study efflux transporter

activities across the blood-placental barrier (Kitano, Iizasa et al. 2002; Lee, Sai et al. 2011). The human BeWo line has been of particular interest because it can be induced to differentiate partially into STB by exposure to forskolin or 5′cyclic AMP analogs (Wice, Menton et al. 1990). However, BeWo cells have proved most useful under conditions in which they form a confluent polarized monolayer on a permeabilized membrane, thereby providing a system to study trans-epithelial transport and metabolism of xenobiotics (Feinshtein, Erez et al. 2013) and small particles of foreign matter (Cartwright, Poulsen et al. 2012). Whether this set-up is valid for mimicking the contact surface between trophoblast and the maternal blood circulation, which, in the placenta, is primarily villous STB, is questionable, however. In any case, the positions of all laboratory choriocarcinoma cell lines in the trophoblast lineage hierarchy remain unclear (Hochberg, Rachmilewitz et al. 1992). Moreover, they are cancer cells that have been maintained for decades in culture and likely possess a genome/epigenome and hence phenotype far removed from the trophoblast lineage from which they were derived.

10.5 Placental Trophoblast Derived from hESC

Xu et al. were the first to show that hESC formed trophoblast when they were exposed to members of the BMP family of growth factors and particularly BMP4 (Xu, Chen et al. 2002). The culture condition used was one standard at the time for ESC, namely DMEM with knock out serum replacement containing low concentrations of FGF2 (4 ng/ml). The cells were grown either on a feeder layer of inactivated mouse embryonic fibroblasts (MEF) or on a Matrigel substratum in medium that had been conditioned by MEF. The BMP, most usually BMP4, was added at concentrations ranging from 1 to 300 ng/ml. In our experiments, the usual concentration used has been 10–20 ng/ml. This differentiation pattern in response to BMP4 became more efficiently directed to trophoblast if FGF2, a growth factor required for maintenance of pluripotency, were excluded from the culture medium (Das, Ezashi et al. 2007; Schulz, Ezashi et al. 2008; Ezashi, Telugu et al. 2012). Under these conditions, differentiation along the main germ line lineages was minimized, and the transformation to trophoblast visible under the microscope as a conversion of the cells to a flatter epithelioid morphology that progressed over time from the periphery of the colonies towards their centers. The cells also began to express a number of transcription factors and cytoskeletal proteins associated with early trophoblast and to down-regulate all the major pluripotency markers, including POU5F1 (OCT4), NANOG, and SOX2. These observations have been essentially replicated by several independent groups of investigators (Douglas, VandeVoort et al. 2009; Erb, Schneider et al. 2011; Marchand, Horcajadas et al. 2011; Sudheer, Bhushan et al. 2012; Li, Moretto-Zita et al. 2013; Warmflash, Sorre et al. 2014; Sarkar, Randall et al. 2015). After about 5 days, the colonies also begin to release relatively large amounts of hCG and progesterone into the medium coincident with the appearance of syncytial areas that stain immunohistochemically for the CGA and CGB subunits of hCG (Fig. 10.2/Plate 7) (Das, Ezashi et al. 2007; Schulz, Ezashi et al. 2008; Ezashi, Telugu et al. 2012).

Three other advances have led to improvements in the model. First was the demonstration that trophoblast formation occurred faster and more synchronously if inhibitors of FGF2 and ACTIVIN/TGFB signaling were provided while the ESC colonies were undergoing differentiation (Amita, Adachi et al. 2013). In our laboratory we routinely add two small compounds, the ACTIVIN A signaling inhibitor A83–01, and the FGF2 signaling inhibitor PD173074 (BAP treatment). With these additions almost all the cells in the colonies become positive for the transcription factor CDX2 and the trophoblast marker KRT7 by 48 h (Amita, Adachi et al. 2013; Yang, Adachi et al. 2015). A second improvement to the model came about with the realization that BMP4 need not be present in the medium beyond the initial 24 h (Amita, Adachi et al. 2013), and that its role, rather than directing formation of trophoblast within ESC colonies, is to prime the cells for differentiation (Yang, Adachi et al. 2015). Indeed, differentiation of BMP4-primed cells is only achieved when the inhibitors are present and the

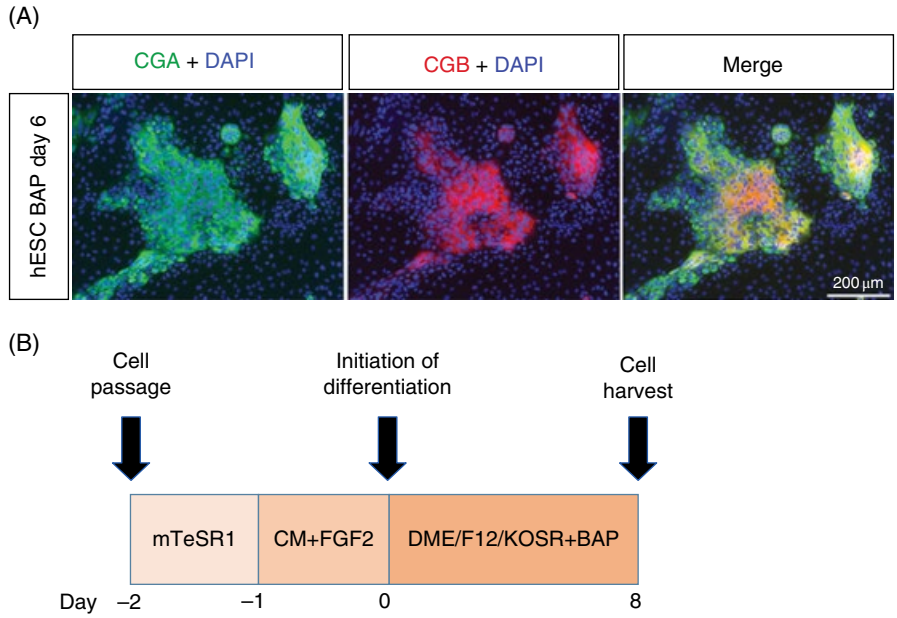

Figure 10.2 ***(Plate 7)*** *STB emerging within colonies of H1 ESC after six days of BAP treatment (A) and illustration of the ESC/BAP differentiation procedure (B). The region shown here has been stained by immunofluorescence localization for CGA and CGB, and by DAPI for nuclear material. The third panel in (A) shows the merged images. These regions of developing syncytium stain for antigens known to be expressed in placental STB. These include CGA and CGB (shown here). CGA generally becomes expressed earlier in the formation of STB than CGB. (See insert for color representation of the figure.)*

signals for maintaining pluripotency blocked. These BMP4-primed cells, called BP cells, can be isolated and remain pluripotent and genetically stable over many passages, but, unlike the parental ESC/iPSC from which they were derived, can be grown on a gelatin substratum. Like mouse ESC, the BP cells can be dispersed to single cells without causing extensive cell death, a property that allows them to be readily propagated, sorted as live cells, efficiently transfected, and readily recovered from cryopreservation (unpublished data). A third advance, which was first achieved with the BP cells, was the realization that it is possible to control the directionality of trophoblast differentiation by altering the relative concentrations of the inhibitors (Yang, Adachi et al. 2015). For example, it has become clear that if the ACTIVIN A signaling inhibitor, A83–01, is absent and only the FGF2 signaling inhibitor, PD173074, present in the medium, the proportion of cells becoming positive for HLA-G (indicative of extravillous trophoblast) is decreased while the proportion of STB formed is increased. Conversely, if the cells are exposed to both inhibitors, there is enrichment of HLA-G-positive cells in the colonies. Although we have no ready explanation for why one of these treatments favors one trophoblast sub-lineage over another, it is now possible to prepare cultures that are enriched for either STB or extravillous trophoblast in less than a week. We suggest that these differentiated pluripotent stem cells will be useful and possibly better alternatives to other trophoblast models used for studying placental toxicology.

ESC and iPSC can also be differentiated to trophoblast when plated onto a suitable semipermeable support. Our laboratory has been using such a system to study the ability of cells within differentiating ESC/iPSC colonies to invade through a substratum of extracellular matrix material, such as Matrigel (Amita, Adachi et al. 2013; Telugu, Adachi et al. 2013). The undifferentiated cells are first cultured on the top of a layer of the chosen matrix material above a membrane containing pores that allow passage of the cells and then placed

under conditions that prompt differentiation to trophoblast. Cells on the undersurface of the membranes are then counted at chosen time intervals and stained for trophoblast markers. It is possible that this trans-well system could also be adapted for transport studies, for example for studying the movement of drugs or other compounds, but we have not, attempted to assess whether or not the cells can form a monolayer exhibiting a trans-epithelial resistance, as do undifferentiated BeWo cells, for example. We also remain doubtful as to whether it would be possible to populate the entire upper membrane surface with STB, the most appropriate cell type for studying transport phenomena as it applies to the placenta. Nevertheless, this trans-well model would seem worthy of further exploration in view of its physiological appropriateness.

In conclusion, therefore, we and others have shown that it is possible to create trophoblast in a dish with relative ease. We suggest that it is feasible to use such cells to study various facets of placental toxicology. Among the most viable applications are studies on the effects of foreign chemicals on placental trophoblast development and gene expression patterns. We suggest that it should also be possible to conduct metabolic and even transcellular transport studies, although the latter may not be straightforward for the reasons given previously.

10.6 Isolation of Syncytial Areas from BAP-Treated H1 ESC Colonies

In our laboratory, we have successfully isolated syncytiotrophoblast from BAP-treated ESC. In this study H1 (WA01) ESC provided the starting material, although the protocol works effectively for iPSC and other hESC. The pluripotent cells are maintained on the chemically defined mTeSR1 medium, which contains added TGFB and FGF2 (Ludwig, Bergendahl et al. 2006), to ensure the cells self-renew and remain undifferentiated, on a Matrigel substratum. To initiate an experiment, cells are passaged into DME/F12 medium with knock-out serum replacement (KOSR) that had been conditioned by mouse embryonic fibroblasts (MEF) to provide a source of TGFB and ACTIVIN and supplemented with low FGF2 concentrations (4 ng/ml). After 24 h, the conditioned medium is replaced with DME/F12/KOSR medium lacking MEF conditioning and minus FGF2, but containing BMP4 (10 ng/ml), A83–01, and PD173074 (BAP treatment) for up to 8 days (Figs 10.2/Plate 7 and 10.3/Plate 8) (Amita, Adachi et al. 2013). Under these conditions, the cells release detectable hCG, progesterone, and placental growth factor (PGF) by d 5, with daily production rising several-fold by d 8, a stage where we generally isolate syncytium.

Rather than using enzymatic methods, colonies are dissociated by using "Gentle Dissociation Reagent™" from Stemcell Technologies. The suspension is then passed successively through a series of nylon screens to provide three cell fractions by size (>70 μm, > 40 μm to < 70 μm, < 40 μm). Here we discuss in detail only the larger > 70 μm and the smaller < 40 μm fractions. The former consists of sheets of cells that stain cytoplasmically for the STB markers (Fig. 10.3C), while the <40 μm fraction is a mixture comprised of single, apparently mononucleated, cells and small cell clumps, some of which stain faintly for CGA subunits and may be fragments of larger syncytial areas (Fig. 10.3B). RNA was isolated from three separate experimental replicates of each fraction, as well as from the initiating H1 ESC (Fig. 10.3A), and subjected to RNAseq, thereby allowing transcript concentrations to be compared.

10.7 Developmental Regulation of Genes Encoding Proteins Potentially Involved in Metabolism of Xenobiotics

RNAseq studies have allowed comparisons to be made of transcript concentrations between undifferentiated ESC and the three size fractions of differentiating trophoblast cells provided in the model. In this review we have chosen to concentrate on the gene expression profile of the large sheets of syncytium that become trapped on the strainer that filters out particles greater than >70 μm in diameter and comprised predominantly

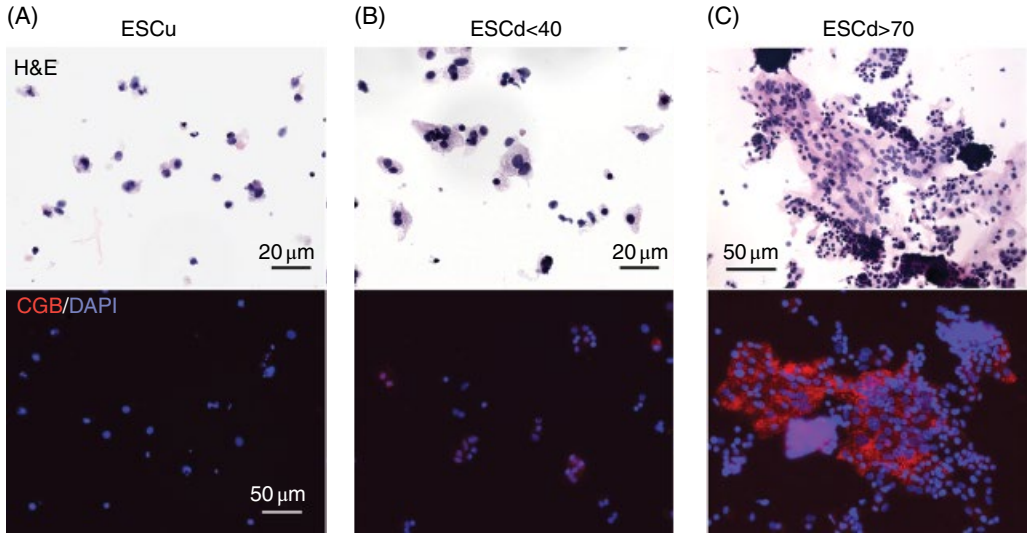

Figure 10.3 ***(Plate 8)*** *Images of three cell populations discussed in the text. (A) H1 embryonic stem cells (ESCu) stained by hemotoxilin/eosin (H & E, top) and stained by immunofluorescence localization of CGB and DAPI. The colonies of ESC were completely dissociated, and dispersed cells collected on a glass slide by using a Cytospin centrifuge procedure (www.thermoscientific.com/en/product/cytospin-4-cytocentrifuge.htmle). (B) Same as above, except the colonies had been differentiated to trophoblast by the BAP procedure (ESCd<40) and fractionated by filtration through a sieve (40 µm mesh size). Note that some fragments of STB are present (top) and that a few clumps stain faintly for CGB (bottom). (C) Same as above, except showing cell fractions retained by a sieve with a mesh size of 70 µm (ESCd > 70). Note the presence of many nuclei in extensive cellular sheets (top), most of which stain strongly for CGB (bottom). (See insert for color representation of the figure.)*

of STB. In doing so, it has been possible to examine several families of genes, some of whose members have a known or suspected function in sensing, metabolism, confining, or transport of foreign chemicals in human tissues. Because of space considerations, the families of genes selected for special discussion have been narrowed to just four: the cytochrome oxidase P450 (*CYP*) family, the very large *SLC* family that encodes membrane transporters, the members of ATP-binding cassette (ABC) transporters, and the *MT* (metallothioneins), whose protein products bind heavy metals. Other families, analyzed but not discussed here, include *HSD* (hydroxysteroid dehydrogenases), GST (glutathione sulfotransferases), and olfactory receptor genes.

10.7.1 Cytochrome P450 Family Members

There are approximately 120 known human CYP genes. The transcripts of 27 of these, including those of three pseudogenes (*CYP51A1P2*, *CYP51A1P1* & *CYP46A4P*) were expressed above background in the >70 µm fraction of theBAP-treated ESC, while only 14 were detectable in the undifferentiated ESC (Fig. 10.4/Plate 9). Eleven CYP transcripts were expressed in both cell types, with the majority upregulated in STB relative to the undifferentiated ESC. Two of them, *CYP11A1* and *CYP19A1* were by far the most strongly expressed members of the entire CYP family (Fig. 10.4/Plate 9). Both were upregulated more than 50-fold compared to their expression in undifferentiated ESC. The enzyme CYP11A1 converts cholesterol to pregnenolone, the key rate limiting step in steroid hormone biosynthesis, while CYP19A1 catalyzes the last steps of estrogen formation. Their low expression in undifferentiated ESC, which produce virtually no steroid hormone, was not, therefore surprising. Analogous data are also observed for *HSD3B1*, which encodes the

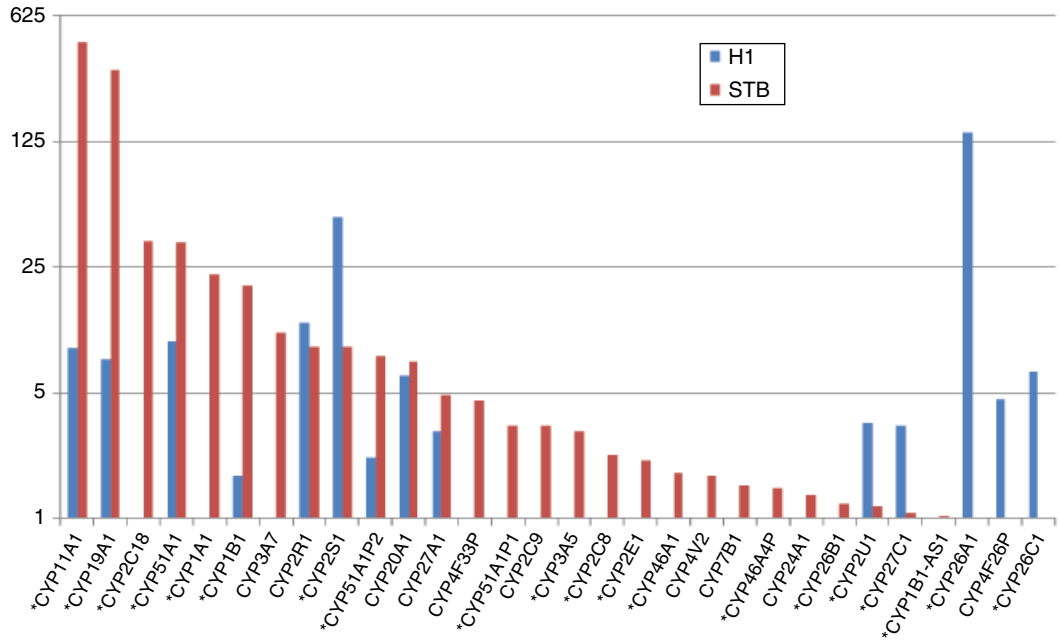

Figure 10.4 ***(Plate 9)*** *Relative expression of CYP genes in H1 ESC (ESCu, blue/dark gray) and STB (ESCd>70, red/light gray). Genes (on the abscissa) marked with an asterisk (*) were expressed differently by the two cell types (FDR < 0.05). Data were obtained by RNAseq on cells at different passage numbers on three separate occasions. The differential expression analysis was performed by using Cufflinks. All values of < 0.01 were recorded as 0.01 to simplify data presentation. Gene expression values (FPKM; fragments per kilobase of exon per million reads) are shown on the ordinate axis. (See insert for color representation of the figure.)*

enzyme that converts pregnenolone to progesterone, and *HSD17B4*, whose protein product catalyzes the conversion of estradiol to estrone (data not shown). The high expression of these four genes, as well as several others, in the STB fraction is consistent with the key role of the placenta, and specifically STB, in maintaining a pregnancy through production of estrogens and progesterone. It is also possible that some of these enzymes can metabolize xenobiotics.

Four CYP450 genes were significantly upregulated in the STB (>70 µm) relative to the cytotrophoblast (<40 µm) fraction (*CYP1A1, CYP1B1, CYP27A1*, and *CYP7B1*), while *CYP2C8* was downregulated (data not shown). Although a precise role for CYP1A1 is unclear, in placenta its gene is upregulated in smokers and induced by some aromatic hydocarbons, such as ones encountered in cigarette smoke (Hakkola, Raunio et al. 1996). CYP27A1 and CYP7B1 are both involved in conversion of cholesterol and cholesterol intermediates to bile acids in liver and play an important role in cholesterol homeostasis (Lorbek, Lewinska et al. 2012), but their function in the placenta is unclear. CYP1B1, like CYP1A1, is capable of NADPH-dependent oxidation of certain aromatic hydrocarbons and aromatic amines (Nebert, Dalton et al. 2004) and also catalyze the conversion of 17 beta-estradiol to catechol estrogens, which are considered potential carcinogens (Liehr 2000). CYP2C8 is an enzyme known to metabolize several xenobiotics (Hakkola, Pelkonen et al. 1998). In liver, *CYP2C8* is inducible by phenobarbital and, as with many other CYP genes, is a possible candidate for up-regulation in response to xenobiotics in STB.

The majority of the *CYP P450* genes discussed previously, including *CYP11A1* and *CYP19A1*, are only weakly expressed in ESC, consistent with the undifferentiated status of these cells and inability to produce

significant amounts of progesterone relative to differentiated trophoblast cells. However, five genes were significantly downregulated in the STB relative to the undifferentiated ESC (*CYP2U1*, *CYP27C1*, *CYP2S1*, *CYP26A1*, and *CYP26C1*). The CYP26 sub-family play a key role in vitamin A metabolism, especially in the hydroxylation of retinoic acid. These enzymes probably have a protective effect against retinoic acid and it ability to direct and, at high concentrations, mis-direct differentiation. CYP2S1 may be involved in the reductive detoxication of aromatic amines, although its normal substrates are unclear (Wang and Guengerich 2013). Similarly, the metabolic roles of CYP2U1 and CYP27C1 are not well established, and they are sometimes classified as "orphan" enzymes. Their association with the pluripotent phenotype is interesting.

10.7.2 SLC Gene Family Members

SLCs comprise a large family of solute carriers, many of which are constitutive to cells undergoing high rates of metabolism. Approximately 272 out of 479 members of the known 492-member SLC gene family were expressed consistently above background in our analyses. Of these, only 22 were differentially expressed (FDR < 0.05) between STB (>70 μm) and cytotrophoblast (<40 μm) fractions (data not shown), while 129 were differentially expressed between the (>70 μm) and the undifferentiated ESC (Fig. 10.5/ Plate 10). Of the most highly expressed SLC genes in in the >70 μm STB fraction, only two (*SLC7A5* and *SLC40A1*) were upregulated, relative to the <40 μm cytotrophoblast fraction. The first of these genes encodes a so-called L-transporter of large amino acids in human syncytial trophoblast (Gaccioli, Aye et al. 2015).

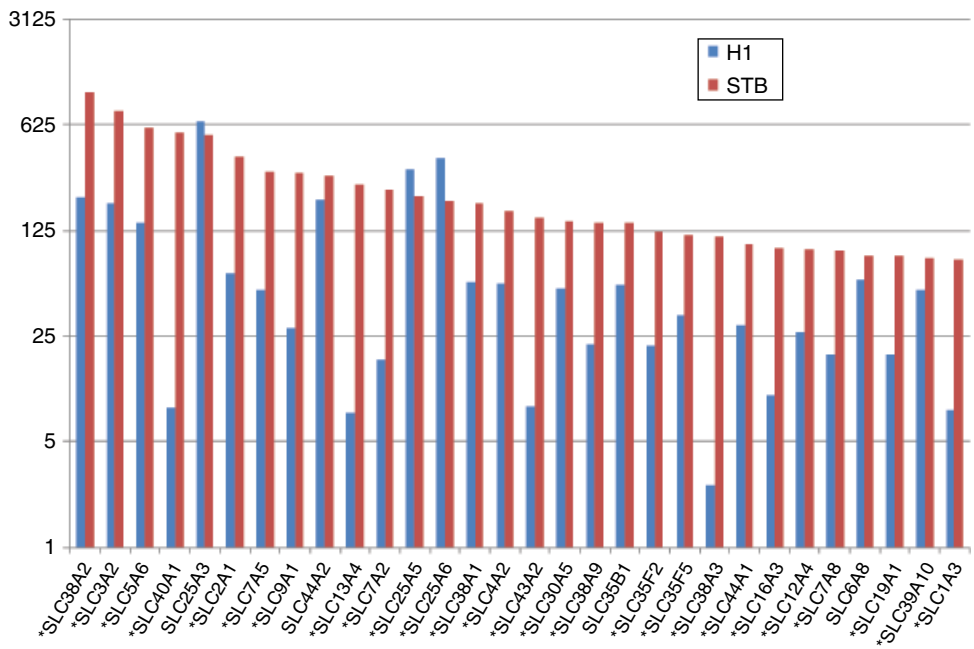

Figure 10.5 ***(Plate 10)*** *Relative expression of SLC genes in H1 ESC (blue/dark gray) and STB (red/light gray). Genes (on the abscissa) marked with an asterisk (*) were expressed differently by the two cell types (FDR < 0.05). Presentation of data is described in Fig. 10.4. However, note the log scale for FPKM values on the ordinate. (See insert for color representation of the figure.)*

It may be a nutrient sensor through the mTOR pathway in the placenta (Aiko, Askew et al. 2014), and binds the thyroid hormones triiodothyronine (T3) and thyroxine (T4) (Geier, Schlessinger et al. 2013). SLC40A1 (ferroportin) is an iron transporter located on the basolateral surface of STB and other epithelia engaged in iron absorption and implicated in transfer of iron from STB to the fetal blood circulation (Koenig, Tussing-Humphreys et al. 2014).

Figure 10.5 (Plate 10) shows relative transcript concentrations for the 30 most highly expressed SLC genes in a comparison of ESC and the >70 µm STB fraction. In general, these genes, including the one encoding the iron transporter SLC40A1, were upregulated in STB compared to ESC. The most abundant *SLC* transcript in the ESC was that of *SLC25A3*, which encodes a mitochondrial phosphate transporter, whose expression altered little upon differentiation.

In general, these data are consistent with the placental trophoblast being a highly dynamic tissue with regard to transport of many kinds of solute. The analyses also re-emphasize an active role of STB in iron metabolism. It remains to be seen whether the expression of any SLC genes is significantly altered when trophoblast is perturbed by foreign chemicals and what sort of consequences would likely result from such environmental insults.

10.7.3 ATP-Binding Cassette (ABC) Transporters

Members of this family of membrane-associated proteins hydrolyze ATP in order to transport a range of different molecules across extra- and intra-cellular membranes. In addition to their normal physiological roles, they are implicated in the absorption, distribution, and elimination of drugs and represent a major defense mechanism against xenotoxic compounds. The ABC family has 49 known members, which are expressed in diverse organs/tissues, including the placenta (Staud, Cerveny et al. 2012). They are categorized into seven subfamilies (ABC A–G) based on structure and substrate specificity of their protein products. In the placenta they are believed play major roles in the active transport of various endogenous metabolites, including sterols, lipids, and nucleotides (Aye and Keelan 2013), but probably also play a role in moving various xenobiotics and drugs out of placental trophoblast (http://nutrigene.4t.com/humanabc.htm).

The best studied of these transporters and the one whose transcripts are most abundant in STB is ABCG2 (Fig. 10.6/Plate 11), which is known as a multidrug resistance-associated protein (MRP) and is sometimes called breast cancer resistance protein (BCRP) because of its ability to pump anti-cancer drugs out of cells and confer resistance to their effects. This high expression in STB is consistent with the observation that placenta ranks first in transcript abundance for *ABCG2* relative to all other human organs and tissues analyzed (Nishimura and Naito 2005). The expression level of *ABCG2* in in the >70 µm fraction, that is STB, was significantly higher ($q = 0.001$) than in the <40 µm fraction, that is predominantly cytotrophoblast (data not shown), and in this sense mirrors what is observed in the placenta, where the the gene product is primarily localized to villous STB (Maliepaard, Scheffer et al. 2001). Reduced *ABCG2* expression has been reported in placenta from pregnancies with intrauterine growth restriction (Evseenko, Murthi et al. 2007) and preeclampsia (Jebbink, Veenboer et al. 2015).

The second most strongly expressed ABC gene in the >70 µm STB fraction, *ABCB8*, has also been implicated in multi-drug resistance, particularly of melanoma cells where it appears to prevent the accumulation of doxorubicin in mitochondria (Elliott and Al-Hajj 2009). The most highly up-regulated gene is *ABCB1*, which is upregulated more than 120-fold in both the stem cell derived cytotrophoblast (ESCd < 40) and STB (ESCd > 70) relative to ESC (Fig. 10.6/Plate 11). ABCB1 has wide specificity that encompasses many amphipathic drugs in addition to endogenously-produced steroids, which may be its physiological substrates (Aye and Keelan 2013).

As it is now widely appreciated that placental ABC transporters play a role in controlling fetal exposure to drugs administered to pregnant women, more information is needed about this class of proteins to minimize

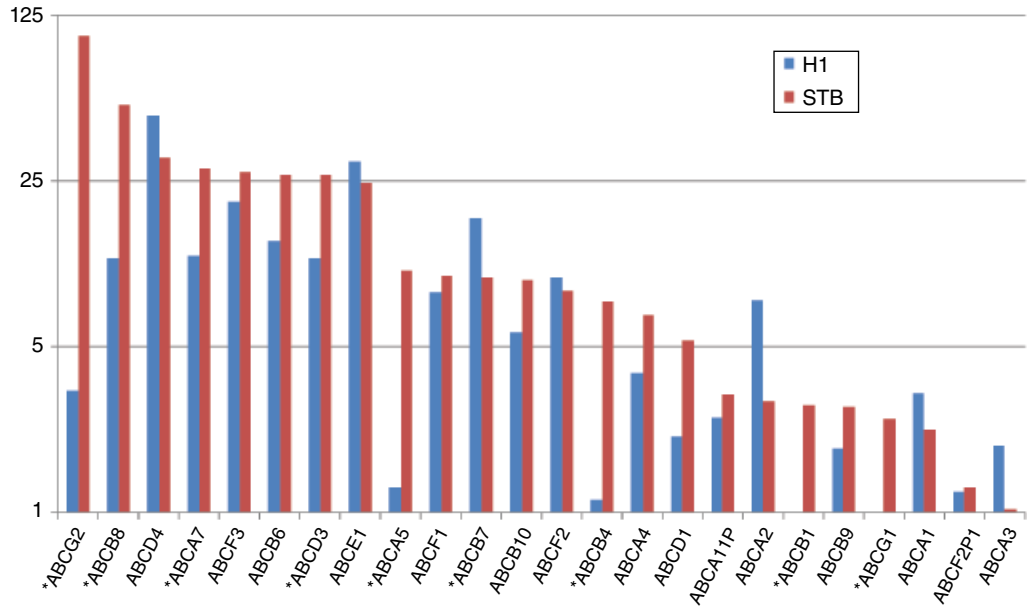

Figure 10.6 ***(Plate 11)*** *Relative expression of ABC genes in H1 ESC (blue/dark gray) and STB (red/light gray). Genes (on the abscissa) marked with an asterisk (*) were expressed differently by the two cell types (FDR < 0.05). Presentation of data is described in Fig. 10.4. However, note the log scale for FPKM values on the ordinate. (See insert for color representation of the figure.)*

the liklihood of the accumulation of a drug in the fetal compartment (Ni and Mao 2011). For example, many of these transporters pursue their normal physiological functions on the basolateral surface of placental STB rather than on the apical surface. Thus, rather than being protective, they may, by happenstance, move drugs entering STB from maternal blood towards the fetus rather than back out into the maternal circulation. Rather than having a protective role, their presence may actually increase the risk to the fetus in women taking certain medications or using recreational drugs. The pluripotent stem cell model, which appears to recapitulate gene expression patterns observed in human placental trophoblasts, may be useful in assessing the rate and directionality of cellular efflux of drugs interacting with ABC transporters and the changing expression of these molecules in response to maternal hormones.

10.7.4 Metallothionein Family Members

Metallothioneins (MT) are rich in cysteine residues (up to 30 %) and bind an array of heavy metals (Lazo and Pitt 1995; Moffatt and Denizeau 1997). There are 18 human isoforms in four classes (I-IV), with classes I and II expressed widely in most tissues. A major role is to regulate intracellular concentrations of the essential elements zinc and copper, but the metallothioneins can also chelate divalent heavy metals such as cadmium, lead, mercury, and platinum, and even trivalent arsenic by virtue of their free thiol groups. Metallothioneins are transcriptionally regulated by both heavy metals and glucocorticoids (Richards, Heguy et al. 1984), and become upregulated in response to heavy metals in cultured choriocarcinoma cells (Lehman and Poisner 1984; McAleer and Tuan 2001). These proteins, thus, play a likely role in metal detoxification, intracellular trace metal homeostasis, and certain stress responses in placenta even though the specialized roles of the individual proteins are not altogether clear.

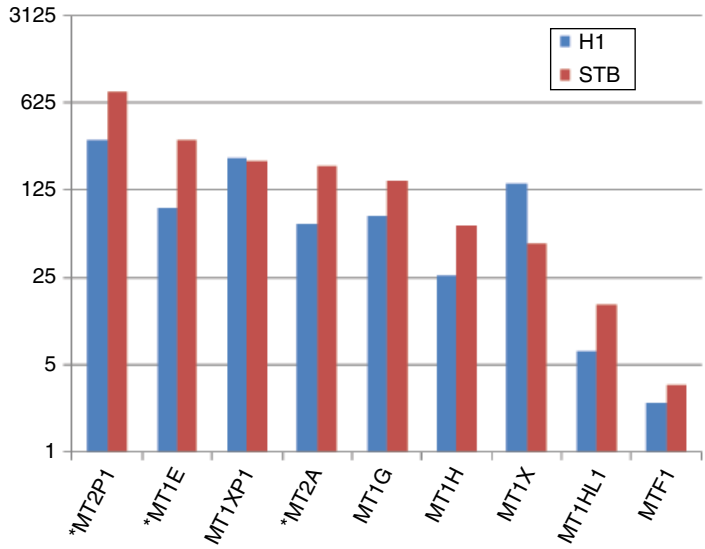

Figure 10.7 (Plate 12) *Relative expression of MT genes in H1 ESC (blue/dark gray) and STB (red/light gray). Genes (on the abscissa) marked with an asterisk (*) were expressed differently by the two cell types (FDR < 0.05). Presentation of data is described in Fig. 10.4. However, note the log scale for FPKM values on the ordinate. (See insert for color representation of the figure.)*

Nine class I and II *MT* genes, including the pseudogenes *MT2P1* and *MT1XP1*, are strongly expressed in ESC and in STB cells derived from them (Fig. 10.7/Plate 12). Most appear to be upregulated in STB relative to undifferentiated hESC, although expression is quite variable, such that only the values for *MT1E*, *MT2A* and *MT2P1* are significantly changed. Class III and IV transcripts are not detectable in either cell type. The same three MT genes are also upregulated in the >70 μm STB fraction relative to the cytotrophoblast (<40 μm) fraction (data not shown). Transcripts for the more restricted class III and IV *MT* genes are not expressed in either the undifferentiated ESC or their differentiated trophoblast derivatives. All these values are likely basal, since the cells have not been deliberately exposed to heavy metals or to other potential inducing agents, such as glucocorticoids prior to transcriptome analysis.

The ESC/BAP model is likely, therefore, to be valuable in mimicking placental responses to heavy metal or arsenic exposure and to elevated stress hormones. The high expression of the two pseudogenes, is puzzling. Whether such transcripts have a regulatory role in gene expression (Tam, Aravin et al. 2008), remains to be determined.

10.8 Concluding Remarks

The conceptus is probably at its most vulnerable to developmental perturbation during the first trimester of pregnancy when the placenta, which is the fetus's first line of defense against a foreign chemical, is becoming established and the orchestrated series of events that create functional fetal organ systems are being launched. However, it is clearly desirable to minimize exposure of the fetus to such compounds throughout pregnancy. An *in vitro* cell culture system that provides functional placental trophoblast and especially STB could, therefore, have considerable currency by providing the potential to forecast the toxicity of drugs and other chemicals to the developing conceptus. The model system we describe, in which human pluripotent stem

cells are primed with BMP4 in absence of FGF2 but in presence of inhibitors of FGF2 and ACTIVIN/TGFB signaling, efficiently provides trophoblast from embryonic or induced pluripotent stem cells. STB and invasive cytotrophoblast are among the cell lineages that emerge, although the two cell types are not organized in a three-dimensional state. The STB fraction can be isolated relatively easily by dissociating sheets of cells and capturing them by gravity filtration. This has allowed STB to be compared with the mononucleated cytotrophoblast within the same colonies. The model will provide a means of assessing whether or not a potential foreign chemical has a subtle but nonetheless subversive influence on the normal differentiation pattern of the stem cells to placental trophoblast by measuring STB areas, analyzing the production of placental hormones and quantifying the ability of cells in the colonies to invade through a layer of extracellular matrix. It will also allow an evaluation of the manner in which a particular xenobiotic is metabolized and whether gene expression is altered by the exposure. Based on baseline data already obtained describing the transcriptome signature of unexposed STB from the differentiating colonies, a number of candidate genes have been identified that may provide sensitive indicators of placental responses to potentially injurious chemicals.

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11

The Effects of Endocrine Disruptors on Mesenchymal Stem Cells

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11.1 Mesenchymal Stem Cells

MSCs have been isolated from a variety of tissues in the human body: bone marrow, adipose tissue, periodontal ligaments, dental pulp, periosteum, synovial membrane, synovial fluid, articular cartilage, muscle tissue, blood vessels, lymphoid organs (thymus, spleen), skin, lung, umbilical cord blood, Wharton's jelly, placenta, amniotic fluid, and fetal tissue (Ksiazek, 2009; Augello, et al., 2010; Mosna, et al., 2010; Hayrapetyan, et al., 2015).

11.1.1 Characterization

The minimal criterion for cells to be termed MSCs has been defined by the International Society for Cellular Therapy (Dominici et al., 2006). The first criterion is plastic-adherence in standard culture conditions (Dominici et al., 2006). Second, MSCs must express surface antigens > 95% CD105, CD73, and CD90 while expressing <2% hematopoietic antigens (CD45, CD34, CD14 or CD11b, CD79alpha, or CD19 and HLA-DR). The third criterion is the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* (Dominici et al., 2006).

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11.1.2 Differentiation

One of the properties of MSCs is the capacity to differentiate into adipocytic, osteocytic, chondrocytic, and other lineages (Pittenger et al., 1999; Vishnubalaji, et al., 2012). This chapter will focus on adipogenesis and osteogenesis, due to the possible impact of endocrine disruptors on these processes as discussed next. It is important to note that in general the stimulation of osteogenesis results in suppression of adipogenesis and vice-versa (Nuttall and Gimble, 2004; James, 2013 Atashi, et al., 2015).

Aspects of the cellular environment can affect MSC differentiation. Such aspects may include the stiffness of growth surface, topography, tension, shape (cytoskeletal organization), and soluble factors in the medium (Kilian, et al., 2010; Steward and Kelly, 2014; Hao et al., 2015; Huang, et al., 2015). Exposure of a cell to a specific microenvironment may induce physiologic alterations and even heritable epigenetic conditioning toward a given lineage fate (Gregory, et al., 2005).

11.1.2.1 Adipogenic

Adipogenic differentiation requires a supportive environment for differentiation and a peroxisome proliferator-activated receptor gamma (PPARγ) ligand to strongly promote differentiation (Janesick and Blumberg, 2011). The necessary environment consists of soluble hormonal stimulus, cell density, spatial cell distribution, and solid components of culture (extracellular matrix). Soluble hormonal stimulus may include insulin, glucocorticoids, and agents that increase intracellular cyclic adenosine monophosphate (cAMP) that act through the insulin-like growth factor 1 (IGF-1) receptor, glucocorticoid receptor, and cAMP-dependent protein kinase, respectively. Once the appropriate environment has been obtained, PPARγ ligands come into play. PPARγ is the master regulator of adipogenesis both *in vitro* and *in vivo* (Rosen et al., 2002; Schug, et al., 2011; Zhang et al., 2013). It has been shown to be both necessary and sufficient for this process. PPARγ expression is first induced by CCAAT/enhancer binding protein (C/EBP)β and δ and then engages in a feed-forward loop with C/EBPα (Rosen et al., 2002; Schug, et al., 2011). RXR-PPARγ heterodimer directly regulates expression of target genes, leading to differentiation of preadipocytes into adipocytes (Schug, et al., 2011).

11.1.2.2 Osteogenic

Runx2 (also called core-binding factor alpha 1 or Cbfa1) is a necessary transcription factor in osteoblastic differentiation as it regulates expression levels of alkaline phosphatase (ALP), osteopontin (OPN), type I collagen, bone sialoprotein (BSP), osteocalcin (OC), and osterix (OX) (Hayrapetyan et al., 2015). Expression of Runx2 is activated by canonical Wnt/β-catenin signaling, bone morphogenetic protein (BMP) signaling, and transforming growth factor beta (TGF-β) signaling (Gaur, et al., 2005; Satija, et al., 2007; James, 2013).

11.1.3 Functions and Activities

MSCs can migrate to sites of injury in response to chemotactic signals and then modulate tissue repair by direct differentiation effects and paracrine signaling effects (Schipani and Kronenberg, 2008; Augello et al., 2010; Maxson, et al., 2012). However, it has been shown that MSCs frequently produce functional improvement with minimal evidence of engraftment or differentiation, indicating that the paracrine effect of MSCs may be more effective in wound healing (Prockop, 2007; Schipani and Kronenberg, 2008). MSCs coordinate wound healing in a paracrine manner by recruitment of other host cells and secretion of growth factors and proteins that enhances repair by stimulating damaged cells to regenerate (Maxson et al., 2012). During

ongoing inflammation, MSCs suppress production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFNγ), enhance production of anti-inflammatory cytokines such as interleukin 10 and interleukin 4, and block T-cell proliferation (Prockop, 2007; Maxson, et al., 2012; Rahimzadeh, et al., 2014). During the proliferation phase of wound healing (up to 2 weeks after injury), MSCs produce vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and platelet derived growth factor (PDGF) and recruit host keratinocytes, dermal fibroblasts, endothelial cells, and stem cells. Up to 2 years into healing, MSCs produce TGF-β and keratinocyte growth factor (KGF) and regulate matrix metalloproteinases (MMPs)/tissue inhibitor of metalloproteinases (TIMP) and collagen deposition (Maxson et al., 2012).

11.2 Endocrine Disruptors

The World Health Organization defines an endocrine disruptor as "an exogenous substance or mixture that alters the function of the endocrine system and consequently produces adverse health effects in an organism or its progeny, or population" (Yoon, et al., 2014). EDCs have a variety of effects on aspects of human development and chronic disease. Chronic diseases focused on in this chapter will be those relevant to MSC derivatives, such as obesity and diabetes.

11.2.1 EDC Major Epidemiologic Associations

11.2.1.1 EDC Association with Obesity

Obesity is increasing rapidly in the United States. In the 1980s, 15% of the population was obese (Janesick and Blumberg, 2011). By 2008, 34% of the population was obese (Janesick and Blumberg, 2011). This more than doubling of the prevalence of obesity was not limited to adults. Even preschool children 2–5 years of age demonstrated a more than doubling in obesity prevalence from 5% in 1976–1980 to 10.4% in 2007–2008 (Thayer, et al., 2012). The recent trend in obesity, particularly in this latter age group, cannot be fully explained by overeating or inactivity, and while genetic variability may explain the propensity toward obesity in some individuals, the increase in the rate of obesity cannot be explained by genetic factors alone (Meggs and Brewer, 2007; Janesick and Blumberg, 2011).

In 2002, Paula Baillie-Hamilton hypothesized that exposure to chemicals such as EDCs may be a new element that is escalating rates of obesity in the last few decades (Baillie-Hamilton, 2002). The time frame makes sense, as the obesity epidemic coincided with the marked increase in use of industrial chemicals in the environment within the last 40 years (Newbold, 2010). In 2006, Bruce Blumberg coined the "obesogen hypothesis," which suggests that EDCs and related chemicals promote weight gain. Because adipocytes increase in number during development and plateau after puberty, EDC induction of weight gain may occur by direct action to enhance hyperplasia in childhood or adipocyte hypertrophy in adults. Alternatively, EDCs may alter the homeostatic regulatory mechanisms for appetite and satiety, alter basal metabolic rate, or shift energy balance in favor of calorie storage (Janesick and Blumberg, 2011; Schug, et al., 2011; Lee, et al., 2014).

More recent studies have focused on prenatal or early life exposure to chemicals, which may affect cells and organs during periods of differentiation and thus alter developmental programming of endocrine controls of metabolism, leading to diseases such as obesity and diabetes later in life (Janesick and Blumberg, 2011; Schug, et al., 2011; Lee, et al., 2014). In addition to modulation of developmental signaling pathways by EDCs, Janesick et al. have suggested that the increasing rate of obesity may be a result of epigenetic

alterations, which can occur quickly, become established within a population, and be transmitted to the next generations (Crews and McLachlan, 2006; Janesick and Blumberg, 2011; Vom Saal et al., 2012). The particular epigenetic changes related to obesity relate to adipogenesis and thus must originate within mesenchymal stem cells, the precursors of the adipocytic lineage (Janesick and Blumberg, 2011). Exposure to EDCs during early life may alter the epigenome of multipotent mesenchymal stem cells, preprogramming them toward the adipogenic lineage (Janesick and Blumberg, 2011; de Cock and van de Bor, 2014). This results in a higher steady state number of adipocytes or adipocyte hyperplasia, which has been demonstrated in obese individuals (Janesick and Blumberg, 2011).

11.2.1.2 EDC Association with Diabetes

Data from the 2005–2008 National Health and Nutrition Examination Survey (NHANES) indicated that an estimated 25.6 million people in the United States >20 years of age had diagnosed or undiagnosed diabetes, with associated direct and indirect medical costs of $174 billion in 2007 alone (Taylor, et al., 2013). In 2008, 347 million people had diabetes worldwide, and diabetes is increasing globally (Kuo, et al., 2013; Rezg, et al., 2014). In addition, diabetes is increasingly diagnosed in individuals earlier in life, including adolescents (Taylor, et al., 2013). These facts have led to an investigation of possible etiologies other than diet, sedentary lifestyle, and genetic factors. One such etiology could be exposure to EDCs as type 2 diabetes, which accounts for 90% of diabetes cases, has been shown to be associated with exposure to certain EDCs in epidemiological studies (Kuo, et al., 2013).

11.2.2 Challenges with Exposure Study Interpretation in Human Subjects

Promising epidemiological data as discussed previously has led to studies of EDC exposure in animal and human subjects. While experimental evidence has overall demonstrated that EDC exposures increase body weight, adipose tissue, and diabetes-related metabolic conditions in animal studies, human evidence has been much more mixed. However, it is very difficult to determine associations between human studies and EDCs due to major methodological differences and complexities, including the fact that obesity itself increases the half-lives of EDCs and weight loss increases serum concentrations of EDCs by release from adipose tissue. Additional important issues to be considered regarding EDC exposure may include nontraditional dose-response dynamics, age at exposure and latency between exposure and possible effect, the importance of considering the effect of combinations of EDCs, and epigenetic effects that span multiple generations (Diamanti-Kandarakis et al., 2009).

11.2.2.1 Nonmonotonicity of EDC Dose-Response Curves

EDCs possess many similarities to endogenous hormones, which explain how EDCs can so effectively alter the homeostasis maintained by these hormones in the body. One shared feature of EDCs and hormones is the ability of EDCs to function at extremely low doses in a tissue specific manner (Schug, et al., 2011). It is well established that natural hormones act at serum concentrations in the picomolar to nanomolar range, and many studies have now documented that EDCs can act in the nanomolar to μM range with some possessing activity at picomolar levels (Vandenberg, et al., 2012). This ability indicates that EDCs have nonmonotonic dose-response curves (NMDRC) (Schug, et al., 2011). NMDRCs are an important challenge as regulatory toxicology assumes monotonicity of dose-response curves, a feature that allows for high-dose testing as the standard assessment of chemical safety (Vandenberg, et al., 2012). Generating predictions about the safety of EDCs by testing at moderate or high doses is not appropriate because in NMDRCs, extremely low doses can have

effects not necessarily predicted by exposures at much higher doses (Schug, et al., 2011; Vandenberg et al., 2012). This type of dose-response is a result of the dynamics of hormone receptor occupancy and saturation and is made possible by structural similarity between EDCs and endogenous hormones (Schug, et al., 2011).

11.2.2.2 EDC Exposure at Critical Developmental Windows and Association with Adult Disease

While EDCs normally demonstrate effects at low doses due to their nonmonotonic dose-response curves, they may have even greater effects at low doses if exposure occurs during critical developmental windows. The developing fetus is not completely protected from the external environment by the placenta (Newbold, 2010). Several EDCs have been demonstrated to cross the placental barrier and accumulate in the embryo or amniotic fluid at detectable levels (Fudvoye, et al., 2014). Increased susceptibility to EDC effects during development is due to the increased dependency on sex steroid and thyroid hormones for maturation of fetus and child (Fudvoye, et al., 2014). The developing embryo or fetus undergoes rapid cell division and differentiation, and if cells are altered due to the effects of EDCs, cells that can differentiate abnormally and pass altered programming to subsequent generations of cells (Crews and McLachlan, 2006; Newbold, 2010; Vom Saal et al., 2012). Fetal development is known to be period of increased sensitivity to injury by chemicals such as EDCs, and endocrine systems continue to mature into childhood and adolescence, resulting in continued susceptibility to chemical exposures (Schug, et al., 2011). These exposures may result in alterations in an increase in adipocyte number and obesity later in life, as discussed previously.

Adults require higher concentrations of EDC exposure to result in toxicity and the effects generally last only as long as the EDC is present (Schug, et al., 2011). In addition to the fact that adult cells do not have the same rapid growth and thus requirement for hormones, adults possess protective mechanisms that fetuses and children have not yet developed. Such mechanisms could aid in negating the effects of the EDCs, including DNA repair mechanisms, competent immune system, detoxifying enzymes, liver metabolism, and mature blood/brain barrier (Newbold, 2010). For the reasons discussed, low dose exposures during development can have sustained effects that last years after the EDC is no longer present and can result in increased risk of disease, such as obesity or diabetes, later in life (Schug et al., 2011; De Coster and van Larebeke, 2012). It is notable that associating effects in humans with previous EDC exposure may be very difficult due to long latency periods between exposure during fetal or childhood development and onset of adult disease. However, a recent study demonstrating a four-fold increase in risk for breast cancer in women exposed in utero to DDT starts to close the gap between exposure and outcome following prenatal experience with EDCs (Cohn et al., 2015).

Furthermore, it is important to keep in mind that the stem cell population of tissues in the adult may behave in much the same manner as described previously for fetal exposures. Thus, the relatively undifferentiated cells that persist and differentiate in adults comprise a vulnerable target for developmental reprogramming by EDCs and other biologically active environmental signaling molecules throughout life. This is a relatively new area of study.

11.2.2.3 Effects of Combinations of EDCs

Studies of combined interventions of the EDCs will not be addressed in this chapter. However, it is important to note that in the environment, humans are exposed to a variety of EDCs with diverse mechanisms of action. It is possible that these compounds could have additive effects (De Coster and van Larebeke, 2012). It is similarly possible that exposure to seemingly antagonistic EDCs could negate their effects, but this does not necessarily decrease the harmful effects of these chemicals (De Coster and van Larebeke, 2012). Many studies have reported complex interactions between compounds, and it is difficult to determine the effects of combinations of any given set of EDCs in the environment.

11.2.3 Mechanisms of Action of EDCs

Structural similarity enables EDCs to mimic natural hormones and act through hormone receptors. EDCs were originally thought to act only through nuclear hormone receptors, including estrogen receptor (ER), androgen receptor (AR), thyroid hormone receptor (TR), progesterone receptor, PPARγ, and retinoid receptor (Schug, et al., 2011). However, EDCs have also been shown to act by nonnuclear steroid hormone receptors such as membrane bound estrogen receptors and membrane bound estrogen GPCR (GPR30), nonsteroid receptors such as neurotransmitter receptors, orphan receptors such as the aryl hydrocarbon receptor (AhR), transcriptional coactivators, enzymatic pathways involved in steroid synthesis, or metabolism, and numerous other mechanisms that affect endocrine and reproductive systems (Schug, et al., 2011; De Coster and van Larebeke, 2012; Vandenberg et al., 2012). EDCs can also directly affect genes and make epigenetic modifications to the germ line that may be transmitted to following generations (Schug, et al., 2011; Vom Saal et al., 2012; Fudvoye, et al., 2014). Such epigenetic modifications may include DNA methylation and histone acetylation (Schug, et al., 2011).

While these are general mechanisms of EDCs, mechanisms specific to organophosphate pesticides, DDT, alkylphenols, bisphenol A, polychlorinated biphenyls, and phthalates will be outlined in the following.

11.3 Pesticides

11.3.1 Organophosphates

The ability of organophosphate pesticides to disrupt acetylcholine signaling by inhibiting acetylcholinesterase (AChE) has made these compounds useful for control of insects in agriculture, personal insect sprays, lawn sprays, and pet shampoos (Hoogduijn, et al., 2006). The organophosphate category represents up to 50% of the use of insecticides worldwide (Lassiter, et al., 2010). Widespread use of these chemicals has resulted in exposure to many, and the Centers for Disease Control and Prevention (CDC) has confirmed that the population of the United States has detectable concentrations of organophosphates in their blood and urine (Meggs and Brewer, 2007). Exposure may occur through residues on food and also as a result of treatment of commercial and residential buildings for pest control (Meggs and Brewer, 2007). However, these pesticides have been recently demonstrated to have other effects, which may categorize them as endocrine disrupting compounds.

11.3.1.1 Cell-Type Specific Effects

It is to be noted that animal studies have demonstrated NMDRCs for chlorpyrifos and malathion (Vandenberg, et al., 2012). Therefore, these compounds and related compounds may have greater effects at lower concentrations, and effects may differ at high and low concentrations.

11.3.1.1.1 Effects on MSCs Bone marrow-derived MSCs (BMSCs) have been shown to express choline acetyltransferase, AChE, and acetylcholine in addition to the nicotinic acetylcholine receptor subunits α3, α5, α7, and the muscarinic acetylcholine receptor 2 (Hoogduijn, et al., 2009). In addition to inhibiting AChE, organophosphate insecticides may also act directly through acetylcholine receptors on BMSCs in a manner similar to nicotine and muscarine. Chlorpyrifos has been demonstrated to increase intracellular calcium concentrations at a speed comparable to nicotine, and chlorpyrifos-oxon has the ability to decrease forskolin-induced cAMP production in a manner similar to muscarine (Hoogduijn, et al., 2009). These effects on calcium and cAMP are notable because calcium and cAMP may initiate signaling pathways that have effects on BMSC proliferation, cytoskeletal organization, and differentiation. Chlorpyrifos-oxon and chlorpyrifos also

induced phosphorylation of extracellular signal-regulated kinase (ERK) 1 and 2, similar to nicotine and muscarine (Hoogduijn, et al., 2009). Phosphorylated ERK has been shown to activate the Fos/Jun transcription complex in many cell types, acting as a regulator of cell proliferation and differentiation. These results suggest that the expression profile of acetylcholine receptors on BMSCs may determine the sensitivity of these cells to EDCs such as organophosphate compounds and that organophosphate compounds such as chlorpyrifos and its metabolites may have effects that alter regulation of BMSC proliferation and differentiation.

11.3.1.1.2 Effects on Adipocytes and Precursors of Adipocytes As discussed previously, endocrine-disrupting compounds such as organophosphate insecticides may play a role in the obesity epidemic. Subpopulations with the highest exposure to organophosphates during childhood have been identified as those with the greatest obesity rates (Lassiter, et al., 2010). Additionally, early-life exposure to low levels of organophosphates has been shown to result in prediabetes, abnormal lipid metabolism, and promotion of obesity (Thayer, et al., 2012). While these organophosphate compounds may not be an etiology for these chronic diseases in all of those affected, they may trigger pathways that result in prediabetes, abnormal lipid metabolism, and obesity in susceptible individuals. We will focus on these pathways in adipocytes, as these are derivatives of MSCs.

In spite of promising epidemiological studies, *in vitro* and *in vivo* studies have been limited and have shown mixed results with regard to obesity-promoting effects of organophosphates on adipose tissue and its component adipocytes. Parathion has been shown to cause a small but significant increase in weight at 0.1 mg/kg dose in male rats, but it has been shown to reduce female rat weight by 4% at 0.1 or 0.2 mg/kg dosing (Lassiter, et al., 2010). In an *in vivo* study of Long-Evans rats treated with chlorpyrifos, the chlorpyrifos-treated subjects demonstrated increased body weight due to an increase in adipose tissue (Meggs and Brewer, 2007). In another study of pregnant rats given 2.5 mg/kg chlorpyrifos per day from gestational day 7 through the end of lactation, male offspring were shown to experience excessive weight gain, reaching levels 10.5% above control rats (Lassiter and Brimijoin, 2008). However, chlorpyrifos was not noted to affect adipocyte proliferation or differentiation in tissue culture studies (Meggs and Brewer, 2007). This lack of effect *in vitro* was further confirmed by Hoogduijn, et al., whose group demonstrated that 0.1, 1, or 10 μM concentrations of chlorpyrifos administered during BMSC adipogenic differentiation did not alter AChE levels or change the intensity of oil red O staining and thus did not have an effect on adipogenic differentiation (Hoogduijn, et al., 2006). In another study of chlorpyrifos effect on 3T3-L1 preadipocytes, a decrease in lipid accumulation was induced (Taxvig, et al., 2012). However, parathion has been shown to decrease lipolysis in adipocytes in a linear concentration-dependent manner (Caley and Jensen, 1973). Thus, parathion could increase triglyceride accumulation in fat tissue which could explain its ability to increase body weight of male rats.

Only one study has reported possible diabetogenic effects of organosphosphates *in vitro*. Specifically, chlorpyrifos has been shown to lower leptin, resistin, and adiponectin release (Taxvig, et al., 2012).

In an *in vivo* study of the effects of neonatal parathion exposure in rats, parathion was noted to disrupt lipid metabolism in adulthood. In males on a normal diet which were exposed to 0.2 mg/kg parathion, a significant decrease in circulating adiponectin was noted. No adiponectin effect was induced by parathion in female subjects. Additionally, in subjects given normal diet, TNF-α levels were increased in the mesenteric fat pad in females and in the inguinal fat pad in both females and males. This study also demonstrated an uncoupling of the relationship between body weight and leptin in male and female subjects, which is important because leptin levels represent a major control point for lipid metabolism. Additionally, the decrease in serum adiponectin levels in males and increase in TNF-α levels in adipose tissue of both sexes were consistent with inflammation of adipose tissue (Lassiter, et al., 2010). Adiponectin is predominantly expressed by adipocytes and has been shown to increase glucose utilization and fatty acid oxidation in muscles and to increase sensitivity to insulin in the liver (Schmidt, et al., 2012). Depression of adiponectin is seen in prediabetes, linked to

decreasing insulin sensitivity and also to dyslipidemia. Additionally, a chronic adipose inflammatory state is seen in diabetes and obesity (Lassiter, et al., 2010).

See Figure 11.1(A) (Plate 13) for a summary of effects of organophosphate compounds on MSCs and cells of the adipocytic lineage.

11.3.1.1.3 Effects on Osteoblasts and Precursors of Osteoblasts A study of the effects of organophosphates on BMSCs demonstrated that a 10 μM concentration of chlorpyrifos reduced AChE activity to 59% of control levels (Hoogduijn, et al., 2006). The metabolized product of chlorpyrifos, chlorpyrifos-oxon, reduced AChE even further to 7% of control levels at its 10 μM concentration. No chlorpyrifos-induced changes in cellular morphology, survival, or proliferation were noted. When chlorpyrifos treatment was administered during osteogenic differentiation, chlorpyrifos (1 μM) increased Stro-1 expression but did not affect expression of CD29, CD44, CD73, CD105, and CD166. MSC differentiation normally leads to a decrease in expression of these MSC markers, suggesting that chlorpyrifos had an effect on osteogenesis (Hoogduijn, et al., 2006). The effect of chlorpyrifos to inhibit BMSC osteogenic differentiation was shown to be at least partially mediated through its ability to inhibit AChE, as chlorpyrifos (1 μM) significantly reduced peak AChE activities in BMSCs undergoing osteogenic differentiation to 68% of control levels (Hoogduijn, et al., 2006). Acetylcholine has been shown to reduce ALP activity via nicotinic acetylcholine receptors and muscarinic acetylcholine receptors in osteoblasts. Thus, inhibition of AChE and the resulting increase in acetylcholine levels may regulate differentiation of osteoblasts (Sato, et al., 2010). The inhibitory effect of chlorpyrifos (0.1, 1, and 10 μM) on osteogenesis was further confirmed by decreased alkaline phosphatase activity and decreased mineralization after 18 days of osteogenic differentiation. Interestingly, inhibitory effects of chlorpyrifos treatment were shown to be reversible; BMSCs pretreated with chlorpyrifos for 4 weeks prior to osteogenesis maintained ALP activity at vehicle-treated levels (Hoogduijn, et al., 2006). This may be explained by the ability of the cell to compensate and increase production or activity of AChE over this pretreatment time period. In summary, chlorpyrifos and related organophosphate compounds may affect bone development and remodeling via effects on cholinergic signaling pathways in BMSCs. These effects are summarized in Figure 11.1(B) (Plate 13).

11.3.1.2 Molecular Effects

While the hormonal mechanisms of action of organophosphate pesticides remain unresolved, the ability of these chemicals to bind and to have effects on nuclear receptors such as AR and ER in some luciferase reporter-responsive assays raises this possibility. Because these effects were assessed in reporter assays, it has not been determined whether these effects occur specifically in MSCs or MSC lineages. However, MSCs and the cells of MSC lineage contain androgen receptors, and thus, these effects may be relevant to the impact of endocrine disrupting compounds on these cells.

Fenitrothion, fenthion, ethyl 4-nitrophenyl phenylphosphonothioate, and parathion have been shown to block DHT-dependent AR activity (Tamura, et al., 2001; Kitamura, et al., 2003; Xu, et al., 2008). Chlorpyrifos has been shown to inhibit testosterone-induced AR effects (Viswanath, 2010). Antiandrogenic activity of fenthion was notable for its similarity in magnitude to that of flutamide, a specific androgen-receptor antagonist (Kitamura, et al., 2003). Fenthion was also found to have the capacity to block androgen-dependent tissue growth in rats (Kitamura, et al., 2003). Other organophosphate pesticides including fenthion sulfoxide, fenthion sulfone, fensulfothion, trichlorfon, and malathion did not show any antiandrogenic activity (Kitamura, et al., 2003; Kjeldsen, et al., 2013). Malathion has also not been shown to have AR agonist activity (Kjeldsen, et al., 2013).

With regard to ER activity, fenthion was not found to have estrogenic or antiestrogenic activities (Kitamura, et al., 2003). Malathion has been shown to induce ER transactivity (Kjeldsen, et al., 2013).

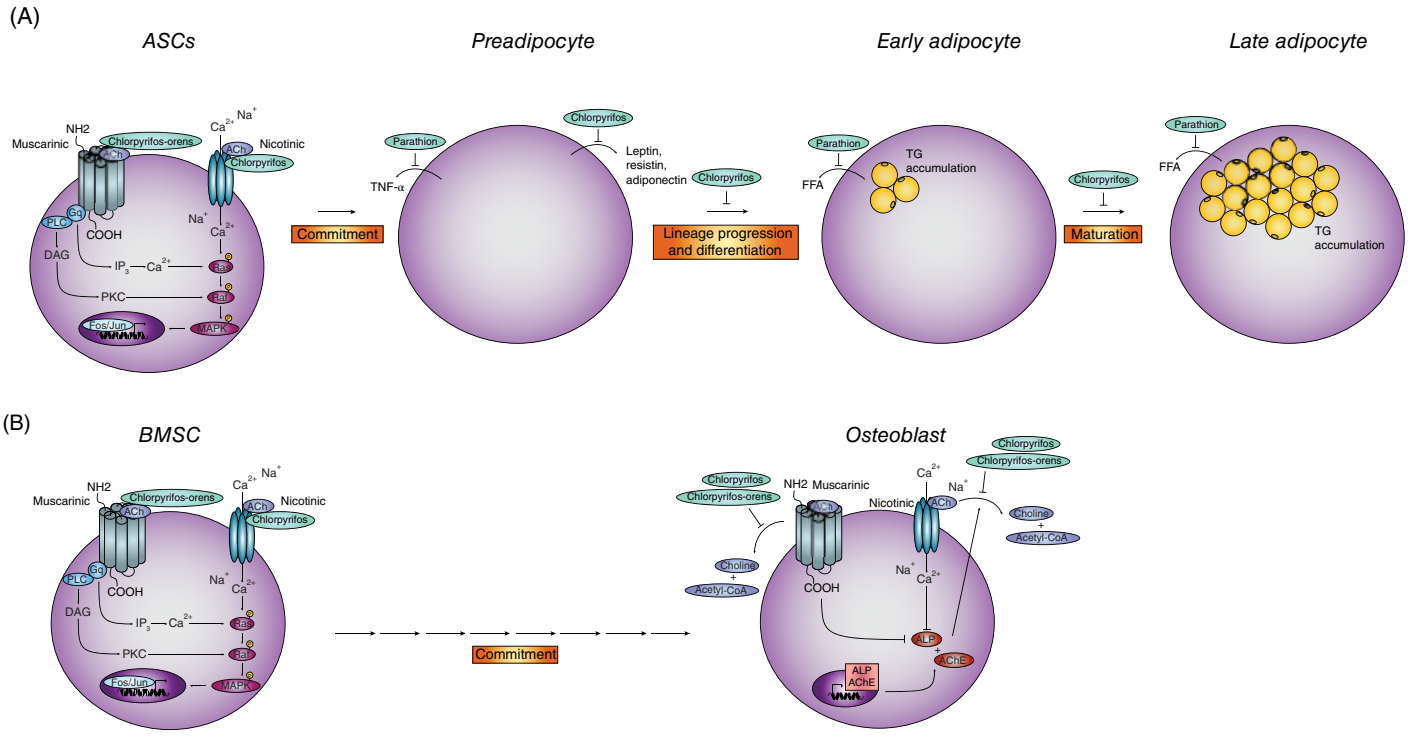

Figure 11.1 ***(Plate 13)*** *(A) The effects of organophosphate pesticides on ASC differentiation into adipocytes. (B) The effects of organophosphate pesticides on BMSC differentiation into osteoblasts. (See insert for color representation of the figure.)*

11.3.2 DDT

Large-scale industrial production of 1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane (DDT) began in 1943 (Yang, et al. 2006). It was widely utilized on agricultural crops and for vector control of insects that carry infectious diseases such as malaria and typhus. During this time, DDT accumulated in soils and sediments (Annamalai and Namasivayam, 2015). As recent as the early 1960s, approximately 400,000 tons of DDT was used annually, and between 1950 and 1970, nearly all Western communities were exposed to DDT in their diet or in the environment (Beard, et al., 2000; Yang, et al., 2006). Additionally, DDT and its metabolites were shown to be transferred from the placenta and breastmilk to fetuses and infants (Yang, et al., 2006). It has been shown that DDT breaks down to p,p'-DDE (bis[4-chlorophenyl]-1,1-dichloroethene) and DDD (1,1-dichloro-2,2-bis [p-chlorophenyl]-ethane) (Beard, et al., 2000; Yang, et al., 2006). DDD is rapidly detoxified and excreted from the human body, but DDT and DDE are stored in fat and metabolized very slowly (Beard, et al., 2000; Yang, et al., 2006). The United States and other Western countries banned DDT in the 1970s due to concerns regarding bioaccumulation in the food chain and adverse health effects on wildlife. Restrictions on DDT use in developing countries were not common until the 1980s (Yang, et al., 2006). However, DDT continues to be utilized in South America, Africa, and Asia for control of malaria (Lee, Porta, et al., 2014).

11.3.2.1 Cell-Specific Effects

Cell culture experiments have determined that DDT and DDE possess NMDRCs. These results were further confirmed by animal studies that showed a NMDRC for DDT. A subset of studies has provided suggestive evidence for nonmonotonic relationships between p,p'-DDE and human health endpoints (Vandenberg, et al., 2012). Such endpoints will be addressed in the following.

11.3.2.1.1 Effects on Adipocytes and Precursors of Adipocytes Organochlorinated pesticides such as DDT and its metabolites have been reported to be positively associated or not associated with obesity (Tang-Peronard, 2011; Dirinck et al., 2014). Inverse associations with obesity have not been reported (Lee, Porta, et al., 2014). Prenatal DDE exposure has also been determined to have a positive association with body mass index (BMI) at later age in multiple cohort studies (de Cock and van de Bor, 2014). Additionally, in the most recent National Toxicology Program Workshop Review, strong positive correlations of diabetes with DDE have been noted in studies such as the most recent National Toxicology Program Workshop Review (Thayer et al., 2012; Taylor et al., 2013; Dirinck et al., 2014). Therefore, epidemiologic studies overall suggest a role of DDT in promotion of obesity and diabetes.

While there are many epidemiologic studies, there are limited *in vitro* studies of the effects of DDT on MSCs and preadipocytes. In a study by Strong, et al., BMSCs exposed to DDT demonstrated enhanced adipogenic differentiation through an ER-dependent pathway, confirmed by increased expression of glucose transporter 4 (GLUT4), lipoprotein lipase (LPL), PPARγ, and leptin (Strong, et al., 2015). In the Moreno-Aliaga, et al. study, a concentration-dependent increase in adipogenesis was induced when 3T3-L1 preadipocytes were treated with 1–50 μM concentrations of p,p'-DDT, the major component of the DDT mixture (Moreno-Aliaga and Matsumura, 2002). The p,p'-DDT-treated 3T3-L1 cells demonstrated a concentration-dependent increase in the nuclear levels of PPARγ and C/EBPα, which are major players in adipogenesis as described previously. The 20 μM p,p'-DDT-treated cells also showed an increase in binding activity of C/EBPα to its DNA response element, further emphasizing the effect of p,p'-DDT on promotion of adipogenic differentiation (Moreno-Aliaga and Matsumura, 2002). C/EBPα has been shown to bind the promoter and activate transcription of adipocyte genes including aP2, phosphoenolpyruvate carboxykinase (PEPCK), leptin, and GLUT4. While p,p'-DDT increases expression of PPARγ, it is unlikely to act as a direct ligand of PPARγ (Moreno-Aliaga and Matsumura, 2002; Taxvig, et al., 2012). Additional data from this study demonstrated that p,p'-DDT treatment did

not result in changes in expression or binding activity of C/EBPβ or C/EBPδ, so these are not likely involved in mediated the adipogenic effects of p,p'-DDT (Moreno-Aliaga and Matsumura, 2002). In another study in 3T3-L1 preadipocytes, p,p'-DDE was noted to have no effect on lipid accumulation (Taxvig, et al., 2012).

In another study of DDE effects on 3T3-L1 adipocytes, DDE exposure at 2 and 20 μM concentrations was not shown to affect adipogenesis, insulin-stimulated fatty acid uptake, or lipolysis (Howell and Mangum, 2011). The difference between this study and the studies by Strong, et al. and Moreno-Aliaga, et al. may be explained by the fact that this study utilized DDE, not DDT. There may be a difference in adipogenic effect between the precursor and its metabolite. However, exposure to DDE was shown to significantly increase release of leptin, resistin, and adiponectin from mature adipocytes and also to increase resistin and adiponectin expression (Howell and Mangum, 2011; Taxvig, et al., 2012). This finding suggests that DDE exposure may promote adipocyte dysfunction characteristics that are often associated with obesity and type 2 diabetes (Howell and Mangum, 2011).

In addition to effects on differentiation, DDE exposure to human preadipocytes for 48 hours has been shown to result in a significant increase in preadipocyte proliferation (Chapados, et al., 2012). Thus, DDT and its metabolite DDE have been shown to affect differentiation, proliferation, and diabetogenic characteristics of preadipocytes. Effects of these compounds on preadipocytes and mature adipocytes are summarized in Figure 11.2 (Plate 14).

11.3.2.1.2 Effects on Osteoblasts and Precursors of Osteoblasts Only one *in vitro* study has been performed to assess possible effects of DDT and its metabolites on bone. In this study, BMSCs exposed to DDT demonstrated enhanced osteogenic differentiation through an ER-mediated pathway, confirmed by increased expression of osteonectin, Runx2, and c-Fos (Strong, et al., 2015). The results of this study are shown in Figure 11.2(B). Further research will need to be performed to verify the osteogenic effects of DDT.

11.3.2.2 Molecular Effects

p,p'-DDT and o,p'-DDT have been demonstrated to be estrogenic compounds (Lee, Porta, et al., 2014). In addition to classic estrogenic effects, p,p'-DDT and o,p'-DDE have been demonstrated to have low binding affinity for GPR30 (De Coster and van Larebeke, 2012). p,p'-DDE has been reported to be both an estrogen agonist and androgen antagonist (Sonnenschein and Soto, 1998; Yang, et al., 2006; De Coster and van Larebeke, 2012; Mrema, et al., 2013; Lee, Porta, et al., 2014). It has additionally been reported to inhibit aromatase (Yang, et al., 2006).

In addition to its ER-mediated effects, DDT acts through ER-independent pathways. In Human Embryonic Kidney (HEK) 293 cells and MCF-7 breast cancer cells, DDT has been demonstrated to have the ability to initiate crosstalk between p38 mitogen activated protein kinase (MAPK) signaling pathways and transcriptional coactivators such as p300/cAMP response element binding (CREB)-binding protein (CBP) (Bratton, et al., 2009, 2012). In another study, DDT and its metabolites were found to activate the activator protein 1 (AP-1) transcription factor in an ER-independent manner in HEK 293 cells and Ishikawa endometrial adenocarcinoma cells (Frigo, et al., 2002). AP-1 has been shown to recruit the p300/CBP and through this coactivator, promote gene expression (Frigo, et al., 2002; Bratton, et al., 2009).

11.4 Alkyl Phenols and Derivatives

Alkylphenol ethoxylates are toxins widely used as surfactants in domestic and industrial products including detergents, paints, herbicides, pesticides, disinfectants, surface cleaners, emulsifiers, wetting and dispersing agents, antistatic agents, spermicides, pulp and paper, textiles, coatings, lube oils and fuels,

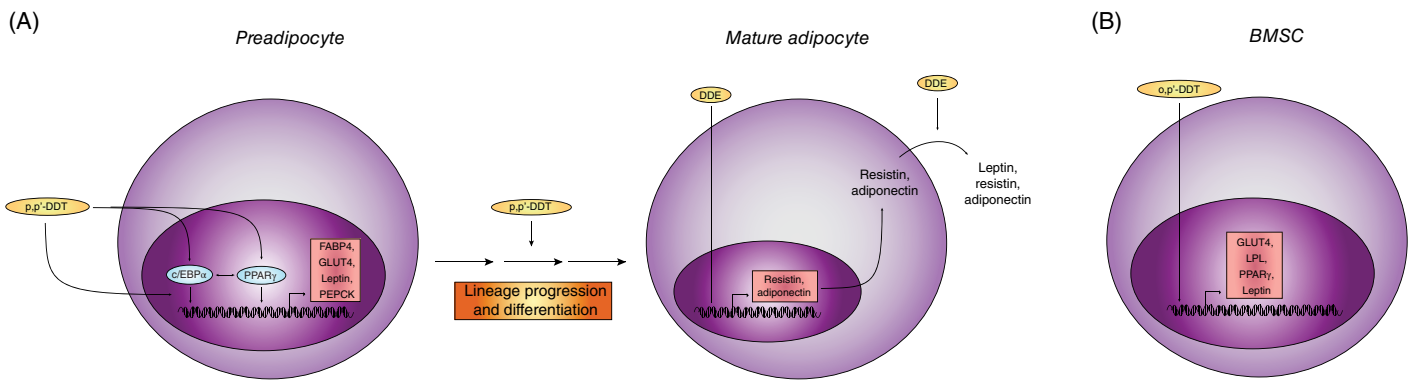

Figure 11.2 ***(Plate 14)*** *(A) The effects of DDT on preadipocyte differentiation into adipocytes. (B) The effects of DDT on BMSC differentiation. (See insert for color representation of the figure.)*

metals, plastics, demulsifiers, and solubilizers (Ying et al., 2002; Rudel and Perovich, 2009; Berge et al., 2012). Approximately 650,000 tons are produced worldwide each year (Yang, et al., 2006). Alkylphenol ethoxylates lose ethoxy groups during biodegradation and become alkylphenols such as nonylphenol (NP), octylphenol (OP), and alkylphenol mono- to triethoxylates (NPE1, NPE2, NPE3). These alkylphenol compounds noted for their stability, persistence, and hydrophobicity, features that have led to accumulation of alkylphenols in wastewater and aquatic environments, volatilization into ambient air, and accrual in soil (Rudel and Perovich, 2009). NP is the most abundant alkylphenol ethoxylate derivative and has been shown to stay biologically active for a longer time period than endogenous estrogen (Annamalai and Namasivayam, 2015). The main pathways of human exposure include dietary and nondietary ingestion, dermal absorption, and inhalation (Rudel and Perovich, 2009). Alkylphenol ethoxylates have been banned in many European countries but continue to be used in the United States and Asia (Sun, et al., 2008).

11.4.1 Cell-Specific Effects

It is to be noted that cell culture experiments have demonstrated nonmonotonic dose-response curves for octylphenol and nonylphenol. Animal studies have confirmed NMDRCs for nonylphenol ethoxylate and octylphenol (Vandenberg, et al., 2012).

11.4.1.1 Effects on Adipocytes and Precursors of Adipocytes

The effects of NP and OP on adipogenesis remain unclear. One *in vitro* study demonstrated that 0.05–25 μM NP suppressed 3T3-L1 preadipocyte differentiation (Pereira-Fernandes, et al., 2013). However, another study showed that 4-NP at a concentration of 10 μM induced adipogenesis in 3T3-L1 preadipocytes (Hao, et al., 2012). In this study, glycerol-3-phosphate dehydrogenase activity, PPARγ expression, and expression of aP2 and LPL were induced by 4-NP (Hao, et al., 2012). The Hao, et al. study also tested the effects of 4-NP (0.5 mg/kg) exposure on C57BL/6J male mice. In the 4-NP-treated mice, expression of PPARγ, aP2, and LPL increased in adipose tissue (Hao, et al., 2012). It has been demonstrated that 4-NP and 4-OP do not have the ability to accelerate terminal 3T3-L1 adipocyte differentiation and even caused a decrease in triglyceride content of adipocytes (Masuno, et al., 2005). However, it is to be noted that the concentrations used in the Masuno et al. study were 45 μM for 4-NP and 4-OP, which are high concentrations and may not be indicative of their effects at lower concentrations due to NMDRC. Based on these studies, it is possible that 4-NP does play a role in induction of adipogenic differentiation, but further testing will be required to verify this fact. There is only one study of 4-OP exposure, so data is too limited to draw any possible conclusions.

Only one study has demonstrated possible diabetogenic effects of alkylphenols thus far. OP has been shown to stimulate expression of resistin in 3T3-L1 preadipocytes by ER and ERK-mediated pathways (Lee, et al., 2008). Resistin is a hormone specific to adipocytes, which can result in insulin resistance and reduce adipocyte differentiation. This effect was also present *in vivo* (Lee, et al., 2008).

Other *in vivo* studies have focused on perinatal exposure to alkylphenols. In a study of perinatal exposure of 4-NP, offspring were shown to have increased body weight, fat mass, serum total cholesterol, and glucose levels with the stronger effect in female offspring at 0.25 mg/kg (Hao, et al., 2012). In another study, serum total cholesterol and leptin levels, quantity and size of fat cells, sterol regulatory element binding protein 1 (SREBP-1) and PPARγ gene expression in adipose tissue, and LPL expression were increased in rat offspring by 4-NP perinatal exposure at 5, 25, and 125 μg/kg/day concentrations. Offspring of 4-NP-exposed first and second generations demonstrated a significant increase in body weight and organ coefficient of adipose tissue in a dose-dependent manner, indicating that weight gain was a result of adipose tissue accumulation.

Interestingly, 4-NP exposure dose-dependently reduced expression of ERα in adipose tissue of both generations of offspring. Deletion and downregulation of ERα have been reported to increase adiposity in mice and to increase fat cell proliferation and differentiation (Zhang, et al., 2014).

See Figure 11.3(A) (Plate 15) for effects of alkylphenols on cells of the MSC adipocytic lineage.

11.4.1.2 Effects on Osteoblasts and Precursors of Osteoblasts

In vitro studies of the effects of NP and OP exposure have shown that these alkylphenols exhibit no effect or an inhibitory effect on osteoblastic differentiation, proliferation, and mineralization (Kanno, et al., 2004; Hagiwara, et al., 2008; Abnosi, et al., 2012). In the study demonstrating an inhibitory effect on differentiation and mineralization of osteoblasts, p-NP also decreased the viability of the cells and caused chromatin condensation, reduction of nuclei diameter, and cytoplasm shrinkage, which may represent induction of apoptosis (Abnosi et al., 2012). Another study showed that incubation of mouse primary calvarial osteoblasts with 4-NP (10^{-5} M and 10^{-6} M) decreased viability of osteoblasts and enhanced phosphatidylserine exposure, a marker of apoptosis (Sabbieti, et al., 2011). Additionally, exposure to 4-NP resulted in upregulation of Bax/Bcl-2 with increase in cleaved caspase 9 and 3, indicating induction of osteoblast death was occurring via a mitochondrial-dependent apoptotic pathway. 4-NP was also capable of increasing cleaved caspase 8 in parallel with truncated active Bid. Apoptotic effects of 4-NP on osteoblasts were shown to overcome 17-β-estradiol-induced survival effects and also to interfere with 17-β-estradiol regulated estrogen receptor expression (Sabbieti et al., 2011). 4-NP at concentrations above 3 μM has been shown to increase intracellular calcium levels in a concentration-dependent manner in human osteosarcoma cells by causing release of calcium from intracellular stores and calcium influx from extracellular space (Wang, et al., 2005). NP also induced calcium-unrelated cytotoxicity in a concentration-dependent manner (Wang, et al., 2005). Therefore, it is likely that NP and OP induce apoptosis in osteoblasts at a wide range of concentrations.

In an *in vivo* study of female mice exposed to OP during perinatal and postnatal periods, a reduction of periosteal bone formation in cortical bone was noted at the femur diaphysis, resulting in a decrease in growth of bone width. Serum OC and ALP positive cells were decreased in the OP-treated mice, indicating that the decrease in bone growth was likely mediated by an OP-induced inhibition of osteoblasts (Kamei, et al., 2008). Based on the cell culture data, this decrease in bone growth may even have been caused by OP-induced apoptosis of osteoblasts.

Figure 11.3(B) (Plate 15) demonstrates effects of alkylphenols on osteoblasts.

11.4.2 Molecular Effects

Molecular effects of NP and OP have been demonstrated in luciferase-reporter response assays. These effects may be relevant to MSCs and their lineages, as the receptors tested are present in these cells.

Multiple estrogenic effects of alkylphenols have been demonstrated. Alkylphenols, including NP and OP, have been demonstrated to bind ERα/ERβ and can initiate transcription of estrogen-responsive genes at a much lower potency than 17-β-estradiol (Bonefeld-Jorgensen, et al., 2007; Sun et al., 2008; Rudel and Perovich, 2009). In addition to direct activation of the ER, alkylphenols can also induce estrogenic effects by other methods (Waring and Harris, 2005). Estrogens are transported in the blood as inactive sulfonate esters, which are synthesized by cytosolic sulfotransferases with the isoform SULT1E1 (Waring and Harris, 2005). Alkylphenols have the ability to inhibit SULT1E1, which inhibits the inactivation of estrogen and therefore increases the level of free active endogenous estrogen (Waring and Harris, 2005). NP has also been shown to have a high binding affinity for the membrane bound estrogen GPCR, GPR30 (De Coster and van Larebeke, 2012).

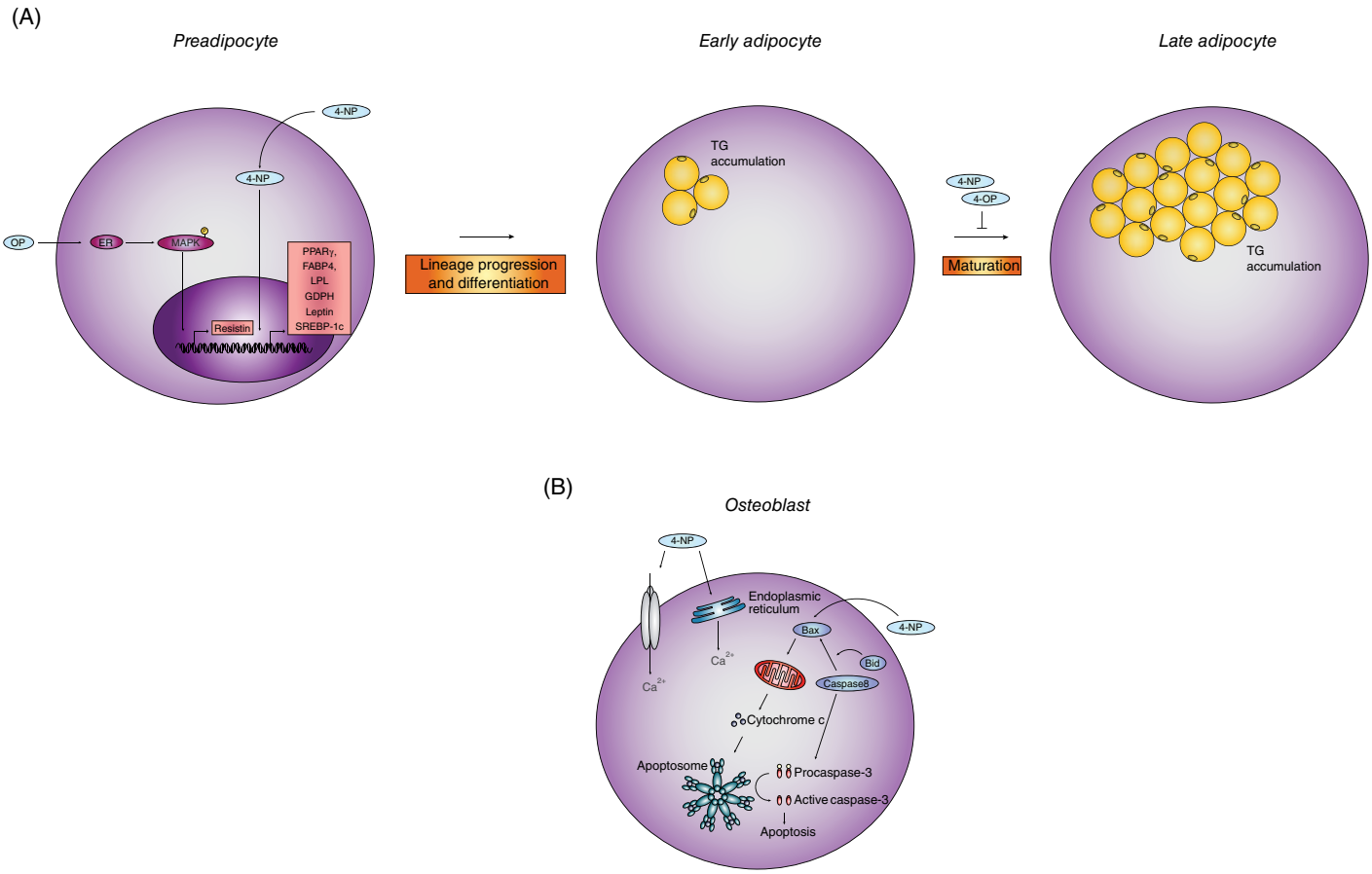

Figure 11.3 ***(Plate 15)*** *(A) The effects of alkylphenols on preadipocyte differentiation into adipocytes. (B) The effects of alkylphenols on induction of apoptosis in osteoblasts. (See insert for color representation of the figure.)*

With regard to the androgen receptor, OP and NP both act as AR antagonists (Xu, et al., 2005; Bonefeld-Jorgensen, et al., 2007). Additionally, NP has been shown to prevent endogenous androgens from binding AR, AR from localizing to the nucleus, AR from interacting with its coregulator, and AR from becoming transactivated (Lee, et al., 2003).

The AhR forms a heterodimer with AhR-nuclear translocator (Arnt) and regulates expression of cytochrome P450 1 (CYP1) and other CYP families. It plays an important role in xenobiotic metabolism and also female reproduction, via activation of CYP19 transcription (Bonefeld-Jorgensen, et al., 2007). In the absence of estrogen, activation of AhR can lead to AhR/Arnt interaction with ERα/ERβ which activates ER-regulated genes. However, in the presence of estrogen, activation of AhR by a ligand inhibits ER binding to its estrogen response element (ERE) and increases the degradation of ER by the proteasome (Bonefeld-Jorgensen, et al., 2007). With regard to AhR activity, NP and OP have both been demonstrated to increase its activity, NP at higher concentrations and OP at lower (10^{-8} M) concentrations. OP also decreases AhR activity at high concentrations (10^{-5} to 10^{-4} M) (Bonefeld-Jorgensen, et al., 2007).

One study indicated the NP and OP had the ability to inhibit aromatase (Bonefeld-Jorgensen, et al., 2007). Another study demonstrated the ability of p-NP to rapidly induce ERK1 and ERK2 phosphorylation, likely via its estrogenic effects (De Coster and van Larebeke, 2012).

11.5 Bisphenol A

BPA is utilized in the production of polymers (polycarbonate, epoxy resins, polysulfone, or polyacrylate), polyvinyl chloride plastics, and flame retardant tetrabromobisphenol-A (Michalowicz, 2014; Rezg, 2014). Epoxy resins form the linings of food and beverage cans while polycarbonate plastics are utilized for baby bottles and containers, such as plastic bottles, plates, cups, goblets, and so on (Michalowicz, 2014; Rezg, 2014). BPA is also found in dental sealants and fillings, sunglasses, thermal papers, CD/DVD discs, medical devices, adhesives, protective coatings, and printer ink (Michalowicz, 2014; Rezg, 2014). Over 6 billion pounds are produced annually, and 100 tons of BPA are released into the atmosphere each year (Rubin, 2011; Rezg, 2014; Gao, et al., 2015). While BPA is a polymer, it degrades into monomeric form over time, especially when exposed to heat, and the monomeric form can leach into adjacent materials, such as packaged foods. Human exposure primarily occurs via ingestion of food containing monomeric leached BPA, and almost all humans are exposed to BPA (Sonnenschein and Soto, 1998; Meeker, et al., 2009; Talsness, et al., 2009; De Coster and van Larebeke, 2012; Michalowicz, 2014; Rezg, 2014). 2003–2004 NHANES data demonstrated that 93% of urine samples (n = 2517) had detectable levels of BPA (Talsness, et al., 2009; Rubin, 2011; Ross and Desai, 2013). Fetuses and children are also exposed to BPA, which has been shown to be present and measurable in breast milk, maternal and fetal serum, amniotic fluid, and placental tissues (Newbold, 2010; Rubin, 2011; De Coster and van Larebeke, 2012; Ross and Desai, 2013; Vaiserman, 2014). The level of BPA in infants and children has been shown to be even higher than in adults (Rubin, 2011; Ross and Desai, 2013).

Concern regarding the possible adverse effects of BPA on human health has resulted in a movement for the removal of BPA from consumer products and its replacement by other compounds. However, some of the replacement compounds, such as bisphenol F and bisphenol S, have also been noted to have endocrine disrupting effects and to be as hormonally active as BPA (Rochester and Bolden, 2015). Therefore, it may be necessary to identify other replacements that are not EDCs.

11.5.1 Cell-Specific Effects

NMDRCs have been demonstrated in cell culture experiments and animal studies for BPA (Vandenberg et al., 2012).

11.5.1.1 Effects on Adipocytes and Precursors of Adipocytes

Epidemiologic studies, including NHANES studies, have demonstrated positive associations between urinary BPA levels and prediabetes, diabetes, BMI, and waist circumference (Talsness, et al., 2009; Rochester, 2013; Fudvoye, et al., 2014; Rezg, 2014; Oppeneer and Robien, 2015). The association with waist circumference indicates that BPA may play a role in both general and central obesity (Rochester, 2013). This section will focus on the effects of BPA that have been identified in adipocytes and their precursors.

In vitro studies of 3T3-L1 preadipocytes have shown that BPA has effects on adipocyte proliferation and adipogenic differentiation, which may support the epidemiologic findings associating BPA with BMI, waist circumference, and obesity. Preadipocytes have been shown to increase proliferation in response to BPA treatment (Ross and Desai, 2013). BPA has been shown to increase adipogenic differentiation of 3T3-L1 preadipocytes to mature adipocytes (Masuno, et al., 2002; Newbold, 2010; Pereira-Fernandes, 2013; Sargis, et al., 2010; Chamorro-Garcia, et al., 2012; Wang, et al., 2013; Boucher, et al., 2014). Additionally, BPA has been shown accelerate terminal differentiation of 3T3-L1 cells to adipocytes (Masuno, 2005) (Fudvoye, et al., 2014). It has been shown that BPA increased triglyceride content of adipocytes, in addition to the expression of LPL and aP2 (Masuno, et al., 2002, 2005; Wang, et al., 2013; Chamorro-Garcia, et al., 2012; Fudvoye, et al., 2014). These effects have been demonstrated to be mediated through the phosphoinositol-3 kinase (PI-3 kinase) and Akt kinase pathways (Masuno, et al., 2005). In addition to LPL and aP2, BPA also has been shown to increase expression of fatty acid binding protein 4 (FABP4) (Chamorro-Garcia, et al., 2012; Fudvoye, et al., 2014; Menale, et al., 2015). BPA-induced expression of aP2, PPARγ, C/EBPα, and C/EBPβ has been shown to occur through a non-classical ER pathway (Boucher, et al., 2014). BPA also has demonstrated the ability to activate the glucocorticoid receptor (GR), which directly promotes adipogenesis and accumulation of lipids primarily through activation of C/EBPβ and C/EBPδ, leading to expression of PPARγ and C/EBPα (Rubin, 2011; Sargis, et al., 2010; Atlas et al., 2014; Boucher, et al., 2014; Fudvoye, et al., 2014). In another study of GR-mediated direct effects of BPA, upregulation of aP2 was noted through an effect on transcription of C/EBP. In addition to this direct GR-mediated effect on adipogenesis, BPA also has GR-mediated indirect effects. Specifically, increased mRNA expression and enzymatic activity of 11beta-hydroxysteroid dehydrogenase 1 (11β-HSD1), an enzyme that converts cortisone to active cortisol, has also been promoted by BPA exposure and interaction with the GR (Boucher, et al., 2014; Fudvoye, et al., 2014; Wang, et al., 2013). Like BPA, cortisol can bind the GR in adipose tissue and promote adipogenesis (Wang, et al., 2013; Atlas, et al., 2014; Boucher, et al., 2014; Fudvoye, et al., 2014).

In addition to acting through PI-3 kinase/Akt, ERR, and GR pathways, BPA has also been shown to upregulate sterol regulatory element binding factor 1 (SREBF1) and mammalian target of rapamycin (mTOR) pathways in human preadipocytes (Boucher, et al., 2014). SREBF1 is a transcriptional activator involved in metabolism of lipids, adipogenic differentiation, and activation of PPARG, the gene that encodes PPARγ (Boucher et al., 2014). The mTOR pathway is associated with adipogenic differentiation, and mTOR activation occurs primarily through PI-3 kinase/Akt (Boucher, et al., 2014). Once activated, mTOR has been demonstrated to activate PPARG directly and also SREBF1, which activates PPARG (Boucher, et al., 2014).

BPA has not been shown to activate or antagonize RXR or PPARγ directly (Sargis, et al., 2010; Chamorro-Garcia, et al., 2012). BPA did induce a weak activation of PPARγ (Pereira-Fernandes, et al., 2013).

In contrast to the effects of BPA demonstrated in preadipocytes and 3T3-L1 cells, a BPA exposure study in C3H/10T1/2 murine mesenchymal stem cells found that BPA either inhibited adipogenesis or had no effect (Biemann, et al., 2012). When cells were exposed to BPA during undifferentiated growth and proliferation intervals, BPA (10 μM) decreased subsequent adipogenic differentiation, adipocyte amounts, triglyceride content, and expression of FABP4, PPARγ2, LPL, and adiponectin (Biemann, et al., 2012). It has been demonstrated in another study of adipose-derived MSCs (ASCs) that BPA (80 μM) can specifically decrease triglyceride accumulation by attenuating LPL gene transcription (Linehan, et al., 2012). BPA also suppressed

aP2 and C/EBPα expression (Linehan, et al., 2012). Notably, this study did not find an effect of BPA on the number of ASCs that committed to adipogenic differentiation (Linehan, et al., 2012). Exposure of MSCs to concentrations of BPA less than 10 μM did not affect differentiation or triglyceride content in either study (Biemann, et al., 2012; Linehan, et al., 2012). In later stages of adipogenesis such as hormonal adipogenic induction and terminal differentiation, BPA was not noted to have an effect (Biemann, et al., 2012).

In other studies on BMSCs, BPA has demonstrated an inability to affect adipogenesis (Chamorro-Garcia, et al., 2012). However, in a study of human ASCs, BPA exposure (100 pm–10 μM) was shown to significantly enhance adipogenesis at 1 μM concentration after 21 days of culture and at 100 nm at 14 days of culture (Ohlstein, et al., 2014). Ohlstein et al. also showed that BPA increased estrogen receptor 1 transcription and that BPA effects on adipogenesis are likely ER-mediated. BPA significantly increased ASC expression of ERα, ERβ, and PPARγ, the master regulator of adipogenesis (Ohlstein, et al., 2014). BPA also increased expression of and temporally accelerated genes of early stage adipogenesis such as dual leucine zipper-bearing kinase, middle stage adipogenesis such as C/EBPα, and late adipogenesis such as IGF-1 and LPL (Ohlstein, et al., 2014). No effect on expression of SREBP1c, aP2, or C/EBPβ was identified (Ohlstein, et al., 2014). In a similar study of BMSCs, BPA-treated cells demonstrated enhanced proliferation through a MAPK-mediated signaling pathway and decreased self-renewal capacity through an ER-mediated signaling pathway (Strong, et al., 2015). Additionally, 1 μM concentration of BPA was demonstrated to enhance adipogenesis of the BMSCs 1.8-fold relative to vehicle with upregulation of the genes encoding leptin, PPARγ, and LPL (Strong, et al., 2015). The ability of BPA to induce adipogenic differentiation was through MAPK and ER-mediated pathways (Strong, et al., 2015). Further research should address the effects of BPA at concentrations less than 10 μM on adipogenesis of ASCs and BMSCs to verify the findings of the Ohlstein, et al. and Strong, et al. studies, respectively. It is possible that BPA may have a biphasic effect on adipogenesis in MSCs, causing an ER-mediated induction of adipogenesis at lower concentrations and lack of effect or inhibition of adipogenesis at higher concentrations through unknown mechanisms.

The association of BPA with diabetes and prediabetes has also been supported by tissue culture data. Interestingly, exposure of 3T3-L1 cells to low doses of BPA has been shown to significantly inhibit insulin-stimulated glucose use, without interfering with adipogenic differentiation (Valentino, et al., 2013). This effect of BPA may be mediated through impaired insulin-activated receptor phosphorylation and signaling (Valentino, et al., 2013). Low doses of BPA have been reported to inhibit the synthesis and release of adiponectin and also to induce release of inflammatory adipokines including interleukin 6 (IL-6) and TNF-α from human adipose tissues (Hugo, et al., 2008; Talsness, et al., 2009; Kidani, et al., 2010; Newbold, 2010; Sargis, et al., 2010; Valentino, et al., 2013; Wang, et al., 2013; Fudvoye, et al., 2014; Menale, et al., 2015). In one study, low dose BPA exposure also induced IFNγ release by preadipocytes (Valentino, et al., 2013). Adiponectin is an adipocyte-specific hormone that normally decreases insulin resistance and tissue inflammation (Fudvoye, et al., 2014). The effect of BPA on adiponectin is noted to be independent of ER or PPARγ-mediated pathways (Kidani, et al., 2010). The Kidani, et al. study demonstrated the ability of BPA to decrease the levels of Akt and phosphorylated Akt, which likely was the cause of the suppression of adiponectin synthesis in this study by preventing insulin from stimulating the PI-3 kinase-Akt signaling pathway needed to stimulate secretion of adiponectin (Kidani, et al., 2010). This is an interesting finding because the Masuno, et al. study demonstrated that BPA acts through PI-3 kinase-Akt signaling to induce terminal differentiation of 3T3-L1 preadipocytes into mature adipocytes (Masuno, et al., 2005). These two studies demonstrate that BPA likely acts through a complex mechanism to stimulate or inhibit PI-3 kinase-Akt signaling, possibly dependent on the stage of adipogenic differentiation or other factors.

Animal studies have supported the obesogenic findings from epidemiologic and tissue culture studies. Both mice and rats exposed prenatally, perinatally, and neonatally to low doses of BPA have experienced increased body weight and increased adipose tissue weight (Miyawaki, et al., 2007; Somm, et al., 2009;

Newbold, 2010; Vom Saal et al., 2012; Pereira-Fernandes, 2013; Ross and Desai, 2013; Wang, et al., 2013; Fudvoye, et al., 2014). It is notable that low concentrations of maternal BPA have been reported to be more effective than high doses of maternal BPA with regard to promotion of weight gain in offspring (Miyawaki, et al., 2007; Ross and Desai, 2013).Offspring exposed to BPA pre- and perinatally have been shown to experience adipocyte hypertrophy and overexpression of genes associated with adipogenesis, including PPARγ, SREBP-1C, LPL, and C/EBPα (Somm, et al., 2009; Fudvoye, et al., 2014). BPA has also been shown to decrease glucose tolerance and increase insulin resistance in adult male mice and male offspring, supporting diabetogenic associations (Batista, et al., 2012; Fudvoye, et al., 2014).

Adipogenic effects of BPA in MSCs and adipocytic lineage cells are shown in Figure 11.4(A) (Plate 16).

11.5.1.2 Effects on Osteoblasts and Precursors of Osteoblasts

In cell culture studies of the effects of BPA on osteoblasts and precursors of osteoblasts, BPA has demonstrated an overall inhibitory effect on osteogenesis. In a study of mouse BMSCs, BPA administration resulted in a significant decrease in calcification and ALP expression (Chamorro-Garcia, et al., 2012). In one study of MC3T3-E1 mouse osteoblastic cells, BPA at 1–10 μM concentrations induced an increase in ALP activity and cellular calcium and phosphorus contents (Kanno, et al., 2004). However, in another study of MC3T3-E1 cells, BPA at 0.5–12.5 μM concentration was shown to suppress ALP activity and bone nodule formation and to downregulate genes involved in osteogenesis including Runx2 and osterix. BPA additionally suppressed Wnt/β-catenin signaling, which is one of the primary signaling pathways involved in osteogenic differentiation, and even upregulated expression of caspase 9, a marker of apoptosis (Hwang, et al., 2013). It should be noted that BPA was not found to have an effect on osteogenesis in human BMSCs (Chamorro-Garcia, et al., 2012; Strong, et al., 2015). Therefore, it is possible that BPA could have differing effects in mouse and human osteoblasts and their precursors, and further research is required to elaborate the effects of BPA on these cells in humans. Figure 11.4(B) (Plate 16) demonstrates the known effects of BPA on osteogenesis in MSCs and osteoblastic lineage cells.

11.5.2 Molecular Effects

BPA has a wide variety of molecular effects. With regard to its estrogenic effects, BPA has been shown to bind and activate nuclear ERα and ERβ, the non-classical membrane-bound form of ER, the ERRγ orphan nuclear receptor, and GPR30 (Matsushima, et al., 2007; Newbold, 2010; Schug, et al., 2011; De Coster and van Larebeke, 2012; Teng, et al., 2013; Delfosse, et al., 2014; Rezg, 2014; Yoon, et al., 2014; Gao, et al., 2015). Although BPA can bind both ERα and ERβ, it has significantly greater binding affinity for ERβ, and higher urinary concentrations of BPA have been associated with increased expression of estrogen receptor 2, the gene encoding ERβ (Melzer, et al., 2011; Alonso-Magdalena, et al., 2012; Yoon, et al., 2014). Additionally, BPA may inhibit ubiquitination and degradation of ERβ, thus increasing ERβ-mediated transcription (Rezg, 2014; Yoon, et al., 2014).The non-classical membrane-bound ER and ERRγ are bound by BPA with particularly high affinity, and BPA has been shown to be at least equal in potency to estradiol with regard to ability to activate responses via newly discovered membrane associated estrogen receptors (Newbold, 2010; Alonso-Magdalena, et al., 2012; De Coster and van Larebeke, 2012; Gao, et al., 2015). Through these membrane associated estrogen receptors, BPA is able to stimulate rapid responses at concentrations as low as picograms per milliliter (Paris, et al., 2002; Alonso-Magdalena, et al., 2012; De Coster and van Larebeke, 2012; Michalowicz, 2014). BPA has also been shown to activate the GPR30-dependent membrane-bound adenylyl cyclase activity (Thomas and Dong, 2006; Gao, et al., 2015). However, while BPA binds many of the same receptors as 17-β-estradiol, the two compounds do not stimulate expression of the same exact suite of genes (Schug, et al., 2011).

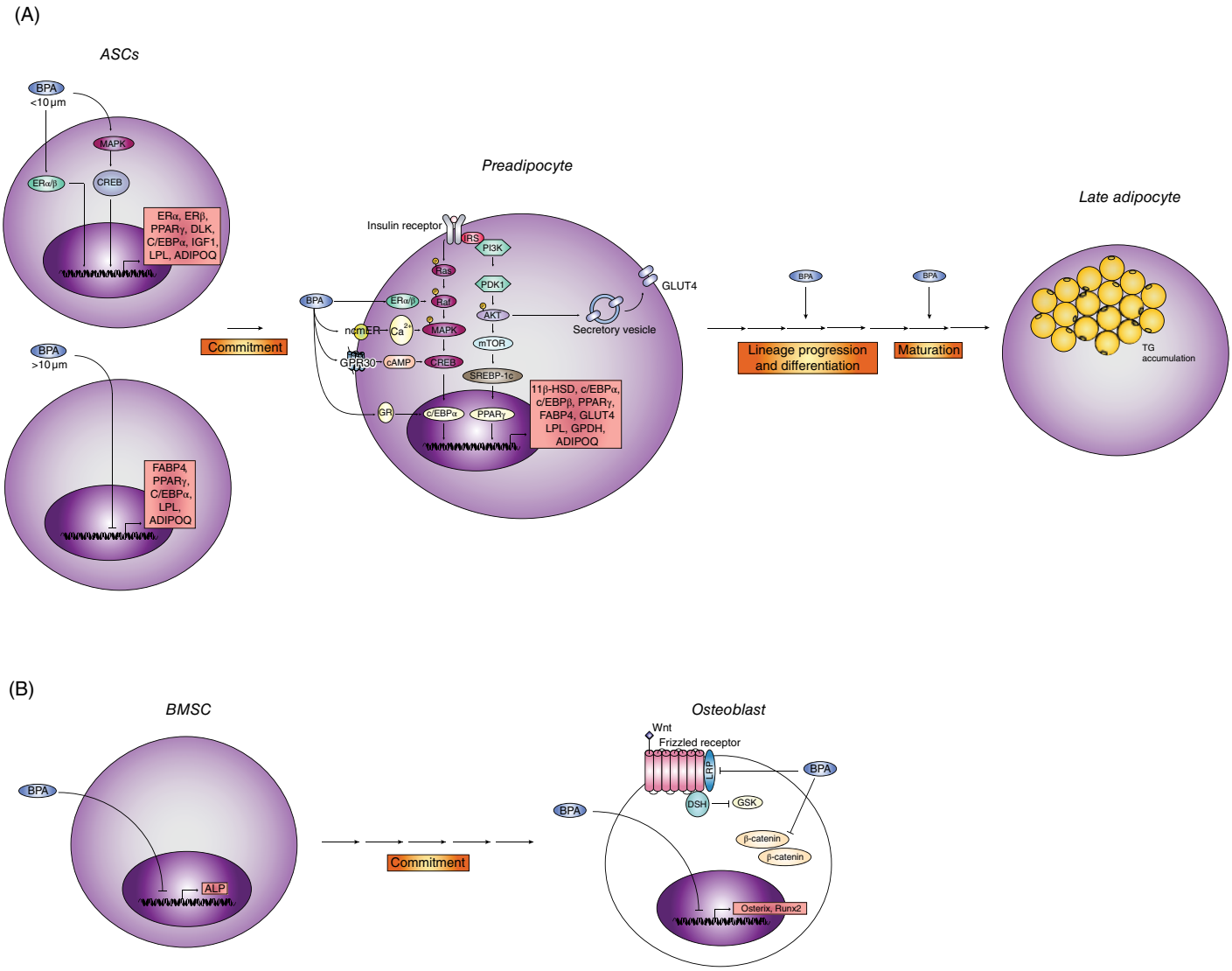

Figure 11.4 ***(Plate 16)*** *(A) The effects of BPA at <10 μM and >10 μM concentrations on ASC differentiation into adipocytes. (B) The effects of BPA on BMSC differentiation into osteoblasts. (See insert for color representation of the figure.)*

With regard to AR, BPA is a well-established antagonist (Paris, et al., 2002; Xu, et al., 2005; Newbold, 2010; Rubin, 2011; Teng, et al., 2013; Delfosse, et al., 2014; Gao, et al., 2015). BPA inhibits multiple steps of AR activation: binding of native androgens to AR, AR nuclear localization, AR interaction with its coregulator, and AR transactivation (Lee, et al., 2003; Teng, et al., 2013; Gao, et al., 2015).

TR have been shown to be a target of BPA in the presence of thyroid hormone (Newbold, 2010; Rubin, 2011; Schug, et al., 2011; Sheng, et al., 2012; Rezg, 2014; Gao, et al., 2015). However, higher concentrations of BPA are required to interact with TR, indicating a lower affinity for TR than for ER (Schug, et al., 2011). Specifically, BPA has been reported to block thyroid hormone from binding to TR or displace iodinated T3 from TR (Waring and Harris, 2005; Rubin, 2011; Delfosse, et al., 2014). The primary method by which low dose BPA inhibits T3/T4 from binding TR is to enhance recruitment of the co-repressor N-CoR/silencing mediator of retinoid and thyroid hormone receptors (SMRT) to TR-β1, which inhibits TR-mediated gene expression (Rubin, 2011; Sheng, et al., 2012). Low dose BPA has also been shown to have the ability to disrupt T3/T4-mediated β3 integrin/c-Src/MAPK/TR-β1 pathways and to involve β3 integrin and c-Src in recruitment of N-CoR/SMRT to TR-β1 (Sheng, et al., 2012).

BPA has also been shown to be an inhibitor of aromatase and an antagonist of AhR (Newbold, 2010; Rubin, 2011). BPA also has the ability to affect cell signaling by increasing calcium intracellularly and causing phosphorylation of ERK and c-Jun N-terminal kinase, and nuclear factor-kappa B (Rezg, et al., 2014).

BPA is notable for its ability to have epigenetic effects and to cause genomic instability (Singh and Li, 2012). With regard to epigenetic effects, BPA has been demonstrated to alter DNA methylation patterns in the germline (Rezg, 2014). It has also been found to cause genetic alterations by inducing DNA adducts, aneuploidy, and mutagenicity in addition to causing improper chromosome segregation and disrupting double-strand break repair (De Coster and van Larebeke, 2012; Rezg, 2014). Epigenetic alterations in ERE sensitivity to estrogen could represent another possible mechanism of action for BPA (Rubin, 2011). These effects and other epigenetic and genomic mechanisms of BPA require further elucidation (De Coster and van Larebeke, 2012).

11.6 Polychlorinated Biphenyls

There are 209 PCB congeners that differ in number and position of chlorine atoms in two benzene rings (Taylor, et al., 2013). Mixtures of PCBs have been in commercial use in the United States since 1929 as heat exchange and dielectric insulating fluid in transformers, capacitors, and as plasticizers in building materials such as adhesives, caulk, ceiling tiles, paints, and sealants (Yang, et al., 2006; Annamalai and Namasivayam, 2015). PCBs have additionally been used for some of these reasons in consumer products in addition to use as flame retardants, hydraulic and heat transfer fluids, vacuum pumps, and gas transition turbines (Rudel and Perovich, 2009). Between 1930 and 1979, over 600 million kilograms of PCBs were used in North America alone (Yang et al., 2006). Restriction of the use of PCBs began in the late 1970s, and their use was banned in the United States in 1976 (Yang, et al., 2006). However, PCBs continue to be major global pollutants due to their thermal stability, resistance to metabolism/degradation, and tendency to accumulate in lipids (Rudel and Perovich, 2009; Annamalai and Namasivayam, 2015). PCBs can bioaccumulate in dietary sources, leading to major exposures through fish, meat, dairy, and processed foods that are primarily contaminated with PCB-153 and PCB-138 (Yang, et al., 2006; Rudel and Perovich, 2009; de Cock and van de Bor, 2014). Of all PCB congeners, PCB-153 has the highest serum level in American adults, likely due to its high dietary presence (Wahlang, et al., 2013). Exposure can also occur through inhalation of atmospheric PCBs, dermal exposure from old electrical appliances, or contaminated sources of soil and water, including wells, surface water, and swimming areas (Yang et al., 2006). As a result, PCBs can be detected in human blood and breast milk (Rudel and Perovich, 2009). In addition to PCBs, risk assessment must take into account the systemic exposure to bioactive metabolites of PCBs including hydroxylated PCBs (OH-PCBs) and MeSO2 (methylsulfonyl)

metabolites (Antunes-Fernandes, et al., 2011; Tehrani and Van Aken, 2014). OH-PCBs have been suggested to have even greater toxicity than parent compounds, and studies in both humans and animals have shown that OH-PCB can be more efficiently transferred across the placenta than parent PCB compounds (Quinete, et al., 2014; Tehrani and Van Aken, 2014). Therefore, the effects of these metabolites will also be discussed next.

11.6.1 Cell-Specific Effects

Cell culture experiments have demonstrated that PCB-74, PCB-118, and Aroclor 1242 are characterized by NMDRCs. Animal studies confirmed that a mixture of PCB and an environmental PCB mixture possess NMDRCs. Additionally, studies provide evidence for nonmonotonic relationships between human health endpoints and PCB-74, PCB-126, PCB-138, PCB-153, PCB-170, PCB-172, PCB-180, PCB-187, PCB-196–203, PCB-196, PCB-199, PCB-201, dioxin-like PCB mix, and non-dioxin like PCB mix (Vandenberg, et al., 2012).

11.6.1.1 Effects on Adipocytes and Precursors of Adipocytes

Several epidemiologic studies have demonstrated positive associations between certain PCBs and increased body weight, BMI, obesity, diabetes, and abnormal metabolism (Mullerova, et al., 2008; Newbold, 2010; Tang-Peronard, et al., 2011; De Coster and van Larebeke, 2012; Thayer et al., 2012; Taylor, et al., 2013; Dirinck et al., 2014; Gauthier, et al., 2014; Lee, 2014; Lim and Jee, 2015. In general, less chlorinated PCBs are those more often associated with obesity (Lee, 2014). In a study of Korean men and women, PCB-28, PCB-138, and PCB-153 demonstrated a significant negative association with adiponectin levels, suggestive of possible induction of diabetogenic phenotype in these individuals (Lim and Jee, 2015).

Studies of the effects of PCBs on adipocytes and their precursors *in vitro* have shown that several PCBs have the ability to promote obesity-related effects in cells (de Cock and van de Bor, 2014. However, only PCB-77 directly stimulates adipogenesis in 3T3-L1 adipocytes include PCB-77 (Arsenescu, et al., 2008; Chapados, et al., 2012; de Cock and van de Bor, 2014). It is notable that the adipogenic effects of PCB-77 require a low dose concentration as high dose concentrations inhibit adipogenesis (Arsenescu, et al., 2008). Low dose PCB-77 was shown to increase expression of PPARγ and its downstream target, aP2 (Arsenescu, et al., 2008). Additionally, glycerol-3-phosphate dehydrogenase activity, a late marker of adipogenic differentiation, was increased by PCB-77 administration (Arsenescu, et al., 2008). The pro-adipogenic effects of low dose PCB-77 are mediated, at least in part, through AhR-mediated pathways (Arsenescu, et al., 2008; Lee, et al., 2014). PPARγ and aP2 expression were not affected by PCB-153 administration (Arsenescu, et al., 2008). In contrast to the positive effect of PCB-77 and the lack of effect of PCB-153, PCB-126 has been shown to inhibit adipogenesis in preadipocytes by an AhR-dependent mechanism (Gadupudi, et al., 2015). This decreased differentiation was associated with downregulation of PPARγ, FABP4, and late adipocyte differentiation genes (Gadupudi, et al., 2015). The contrast between PCB-77 and PCB-126 effects on adipogenesis is representative of the differing effects made possible through AhR agonism, as discussed next.

Leptin is known as an anti-obesity hormone which can prevent accumulation of lipids and promote use of metabolic fuels such as fatty acids rather than storage of fatty acids into triglycerides (Ferrante, et al., 2014). In a study of 3T3-L1 preadipocytes, PCB-101, PCB-153, and PCB-180 were shown to increase lipid content, leptin expression, and reduction of leptin receptor expression and signaling, consistent with "leptin resistance." Leptin resistance in adipose tissue is related to obesity (Ferrante, et al., 2014). Therefore, while PCB-153 and PCB-180 have not been shown to directly promote adipogenesis, they have an indirect effect to induce adipogenic differentiation through leptin inhibition and increase in lipid accumulation.

In multipotent mesenchymal stem cells, studies have also shown effects related to obesity. Obesity-associated inflammatory genes including interleukin 1B, interleukin 1 receptor alpha (IL-1Rα), and interleukin 8 (IL-8)

have been shown to be increased by AhR ligands such as PCB-126 in human ASCs (Kim, et al., 2012). In undifferentiated precursor cells, PCB-153 downregulated IL-6 expression and increased IL-1Rα expression (Kim, et al., 2012). PCB-153 demonstrated no effects on expression in adipocytes (Kim, et al., 2012). PCB-126 exposure resulted in a significant increase in IL-8 and IL-1Rα in undifferentiated cells, and it caused a significant increase in IL-8 in adipocytes (Kim, et al., 2012).

In vitro studies of PCB exposure have demonstrated diabetogenic effects. In 3T3-L1 preadipocytes, low dose PCB-77, PCB-153, and PCB-180 treatments have been shown to result in an AhR-dependent increase in TNF-α expression and IL-6 expression (Portigal, 2002) (Arsenescu, et al., 2008) (Ferrante, et al., 2014). Exposure to PCB-77 at low and high doses (3.4 and 68 μM) has also been shown to cause a decrease in adiponectin expression, another effect which increases insulin resistance (Arsenescu, et al., 2008). When a high concentration of PCB-77 (68 μM) is used, the release of proinflammatory adipokines such as IL-6 from adipocytes is decreased (Arsenescu, et al., 2008). Differentiated adipocytes exposed to PCB-126 underwent a dose-dependent decrease in adiponectin expression (Gadupudi, 2015).

Treatment of mice with PCB-153 has shown an association with increased visceral adiposity, adiponectin, leptin, and resistin (Wahlang, et al., 2013). There was no noted effect on insulin resistance or TNF-α levels (Wahlang, et al., 2013). In a study of PCB-77 and PCB-126 exposure, sustained dose-dependent impairment in glucose and insulin tolerance was noted in low fat-fed mice (Baker, et al., 2013). PCB-77 exposure resulted in weight gain in wild type mice (Arsenescu, et al., 2008). In addition, PCB-77 treated mice demonstrated significant increases in TNF-α expression in adipose tissue and TNF-α plasma concentrations (Baker, et al., 2013). It is to be noted that TNF-α can directly promote insulin resistance via downstream phosphorylation of the insulin receptor docking protein which inhibits Akt/Protein Kinase B phosphorylation and disrupts transport of GLUT4 vesicles to the plasma membrane in skeletal muscle and adipose tissue. Additionally, TNF-α can promote lipolysis or inhibit adipogenesis and thus indirectly increase adipocyte insulin resistance (Baker, et al., 2013). Additionally, PCB-77 treatment resulted in increased IL-6 plasma concentrations (Baker, et al., 2013). The effects of PCB-77 were shown to be AhR-dependent (Arsenescu, et al., 2008; Baker, et al., 2013). In a study of Aroclor 1254-exposed C57B/6 mice, PCB exposure produced hyperinsulinemia and exacerbated whole-body insulin resistance in obese mice without affecting body weight, suggesting that PCB exposure may be a risk factor for diabetes (Gray, et al., 2013).

Effects of PCBs on adipogenesis in MSCs and adipocytic lineage cells are summarized in Figure 11.5(A) (Plate 17).

11.6.1.2 Effects on Osteoblasts and Precursors of Osteoblasts

Several epidemiologic studies of associations between organochlorinated compounds such as PCBs and changes in bone quality/strength or osteoporosis have been reported (Alveblom, et al., 2003; Hodgson, et al., 2008; Paunescu, et al., 2013).

In vitro effects of PCB congeners and mixtures have been extremely limited. In a study of MC3T3-E1 cell exposure to Aroclor 1254, cell proliferation was reduced. Additional effects included increased reactive oxygen species levels, decreased ALP activity and intracellular calcium levels, and increased apoptosis induction as shown by upregulation of Apaf-1 and Bax (An, et al., 2012; Herlin, et al., 2015). A second study further demonstrated reduced expression of osteocalcin, another marker of osteoblast differentiation (Herlin, et al., 2015). These effects of Aroclor 1254 were indicated to be driven by its dioxin-like congener components and thus may be mediated through AhR (Herlin, et al., 2015).

Various *in vivo* effects of PCB congeners and mixtures on osteoblasts and their precursors have been reported. It has been suggested that highly chlorinated PCB mixtures, such as Aroclor 1254, resemble dioxin and thus may demonstrate antiestrogenic effects through the ability to interact with AhR. One study reported that Aroclor 1254 administration affected calcium metabolism and femur structural morphometry in male

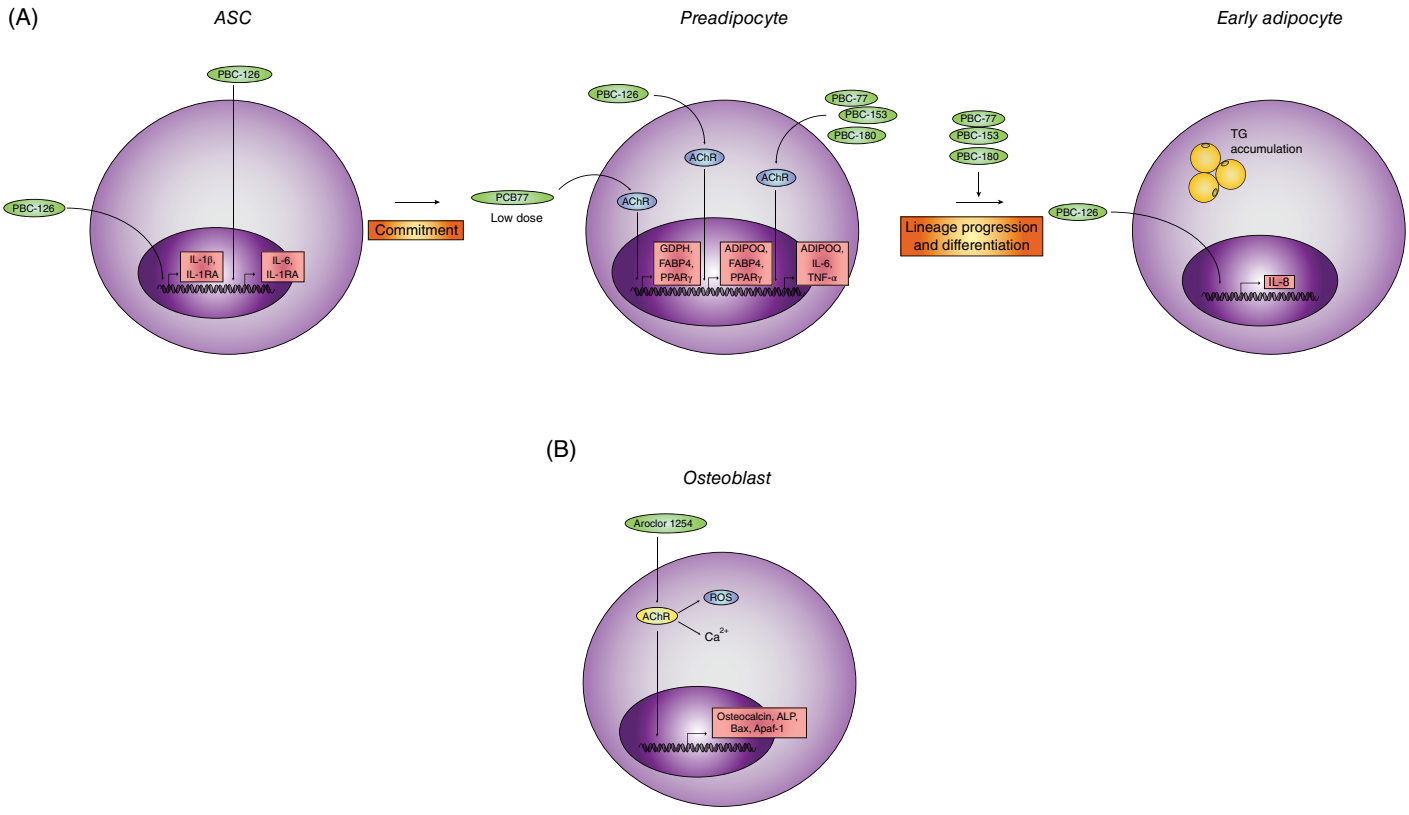

Figure 11.5 ***(Plate 17)*** *(A) The effects of PCBs on ASC differentiation into adipocytes. (B) The effects of PCBs on osteoblasts. (See insert for color representation of the figure.)*

rats, resulting in weaker bone (Andrews, 1989). In a second study, Aroclor 1254 treatment was not demonstrated to alter osteoblastic markers, including serum OC and ALP levels, in intact or ovariectomized female rats (Yilmaz, et al., 2006). However, it produced necrotic areas in vertebral bone in all treated rats (Yilmaz, et al., 2006). Administration of Aroclor 1254 to adult male rats has been shown to decrease ALP activity and collagen levels in the femur, indicating an effect on femoral osteoblasts (Ramajayam, et al., 2007). The study reported that these effects were likely mediated by oxidative stress as the effects were prevented by administration of antioxidant vitamins such as C or E (Ramajayam, et al., 2007). However, the study did not test other possible causes of these effects. Therefore, the effects of Aroclor 1254 may be antiestrogenic but require further studies to clarify their exact mechanism, including studies to assess the possibility of AhR-mediation of effects. Aroclor 1221, due to its low chlorination, has been suggested to be estrogenic. However, in a study of ovariectomized female rats, Aroclor 1221 treatment did not alter serum OC or ALP levels significantly and thus did not affect osteoblast activity (Yilmaz, et al., 2006).

Studies of PCB-126 exposure in rats have demonstrated impaired mineralization in the vertebrae, tibia, humerus, and femur (Lind, et al., 1999, 2000; Alvarez-Lloret, et al., 2009). Sham-operated rats exposed to PCB-126 demonstrated no change in bone mineral density (BMD) or trabecular bone volume of the tibia but did experience an increase in osteoid surface with increased cortical thickness and organic content of the tibia, indicative of impaired mineralization (Lind, et al., 1999). In contrast, PCB-126 exposed ovariectomized rats were found to have decreased length and increased BMD of the tibia, suggesting a possible estrogenic effect (Lind, et al., 1999). In a similarly designed study of PCB-126 effect on humerus and femur of sham-operated and ovariectomized female mice, PCB-126 was shown to reduce bone length, torsional stiffness, and collagen concentration but did not affect BMD (Lind, et al., 2000). Because the PCB-126 exposure resulted in similar changes in sham-operated and ovariectomized mice, there was no estrogenic effect demonstrated (Lind, et al., 2000). Further studies are necessary to confirm or refute the possibility of estrogenic effects of PCB-126 in ovariectomized mice and also to further clarify mechanisms of PCB-126 effects on bone.

In a study of prenatal and perinatal administration of a mixture of PCBs (126, 138, 153, 180), PCBs were noted to have effects on the offspring in adulthood (Cocchi, et al., 2009). Specifically, PCB mixture exposure resulted in a significant decrease in bone mineral content and cortical bone thickness of the tibia at mid-diaphysis in male rats (Cocchi, et al., 2009). These altered parameters were accompanied by a decrease in bone strain strength. Female rats demonstrated no difference between PCB and control treated groups (Cocchi, et al., 2009). In another study of perinatal rat exposure to Aroclor 1254, decreased length and thickness of the femur and tibia with decreased mechanical strength of the femoral neck were seen in offspring at postnatal day 35 (Elabbas, et al., 2011). However, it is important to note that this study demonstrated no treatment-related bone changes in offspring at postnatal days 77 and 350, indicating that changes may be reversible with natural remodeling of bone following discontinuation of the exposure (Elabbas, et al., 2011).

Figure 11.5(B) (Plate 17) demonstrates the effects of PCBs on osteogenesis in osteoblasts.

11.6.2 Molecular Effects

The estrogenic and antiestrogenic effects of PCBs have been explored. PCB congeners 19, 28, 47, 51, 53, 95, 100, 104, 125, 136 exhibited ER-mediated activity (Hamers, et al., 2011). In general, Aroclors 1221, 1232, 1242, and 1248 have shown estrogenic effects while Aroclors 1254, 1260, 1262, or 1268 did not produce this effect. (ATSDR, 2000). PCB-38, PCB-118, PCB-126, PCB-138, PCB-153, PCB-168, PCB-170, PCB-180, PCB-190, and some OH-PCBs have been shown to have antiestrogenic effects (Hamers, et al., 2011; De Coster and van Larebeke, 2012). Most antiestrogenic effects have been shown to be mediated through AhR. However, PCB-138, PCB-153, and PCB-180 have been shown to have antiestrogenic effects mediated through ER (Bonefeld-Jorgensen, et al., 2001; De Coster and van Larebeke, 2012). These PCBs demonstrated

a dose-dependent decrease of estradiol-induced activity in human breast cancer cells and also basal antiestrogenic activity (Bonefeld-Jorgensen, et al., 2001). Interestingly, PCB-100, PCB-101, PCB-138, and hydroxylated PCBs have been noted for their capacity to inhibit SULT 1E1 isoform of hydroxyl steroid sulfotransferase, which normally converts estrogens into their inactive sulfonate esters for transport in the blood (Hamers, et al., 2011). By inhibiting the cytolic sulfotransferase, OH-PCBs increase the amount of endogenous active estrogens (ATSDR, 2000; Waring and Harris, 2005).

With regard to androgen receptor, PCBs have been shown to be AR-antagonists. No PCB congeners have demonstrated androgenic activity (Hamers, et al., 2011). In the presence of DHT, the following PCB congeners have demonstrated complete antagonism of DHT stimulation of AR: PCB-49, PCB-66, PCB-74, PCB-105, and PCB-118 (Schrader and Cooke, 2003; Hamers, et al., 2011). PCBs 19, 28, 31, 42, 47, 51, 52, 53, 80, 95, 100, 101, 104, 122, 125, 126, 128, 136, 138, 153, 156, 168, 170, 180, and 190 were noted to be partial antagonists (Bonefeld-Jorgensen, et al., 2001; Portigal, 2002; Schrader and Cooke, 2003; Mullerova, et al., 2008; Hamers, et al., 2011; De Coster and van Larebeke, 2012). PCB mixtures including Aroclor 1260, 1242, 1254, and 1248 have also been demonstrated to antagonize AR-mediated transcription by DHT (Portigal, 2002).

Some of the PCB congeners have been shown to bind AhR similar to a compound called dioxin. These congeners are thus called "dioxin-like PCBs" and include compounds such as PCB-77, PCB-81, PCB-118, PCB-126, PCB-156, and PCB-169 (Hestermann, et al., 2000; Yang, et al., 2006; Annamalai and Namasivayam, 2015). Once PCBs activate AhR, a heterodimer of AhR and Arnt directly associates with ERα and ERβ, causing recruitment of unliganded ER and coactivator p300 to estrogen-responsive gene promoters, leading to transcription activation. However, this mechanism may also lead to antiestrogenic effects via downregulation of the estrogen receptor, impairment of estradiol binding ER, prevention of ligand-activated ER binding its ERE, and/or induction of cytochrome P450 1A1, 1A2, or 1B1, which metabolize estradiol (Bonefeld-Jorgensen, et al., 2001; Yilmaz, et al., 2006; De Coster and van Larebeke, 2012; Warner, et al., 2012).

PCBs that have been demonstrated to be dioxin-like and possibly antiestrogenic have been associated with upregulation of aromatase expression (Warner, et al., 2012). Such congeners include PCB-74, PCB-105, PCB-118, PCB-138, PCB-156, PCB-157, PCB-158, PCB-167, and PCB-170 (Warner, et al., 2012). OH-PCBs have been shown to act as aromatase inhibitors (Antunes-Fernandes, et al., 2011).

PCBs and PCB mixtures have demonstrated mixed effects with regard to GR. Some have not been shown to alter GR-mediated transcription (Iwasaki, et al., 2002; Portigal, 2002). In the presence of dexamethasone, PCB-42 and PCB-101 did demonstrate increased GR activity (Portigal, 2002; Bovee, et al., 2011). However, GR antagonism in the presence of budesonide has been shown for PCB-47, PCB-92, PCB-100, and even PCB-101 (Bovee, et al., 2011). PCB-101 thus may have GR agonist or antagonistic effects dependent on the dose and possibly other factors. OH-PCBs have shown anti-glucocorticoid effects (Antunes-Fernandes, et al., 2011).

Many PCB congeners and mixtures have been shown to interact with TR. PCBs have not been shown to alter basal TR-induced transcriptional activity. However, PCBs such as 4(OH)-2',3,3',4',5'-pentachloro biphenyl, 4'-OH-PCB-106, 4'-OH-PCB-187, and Aroclor 1254 have been shown to suppress T3-induced transactivation of TR by a mechanism other than ligand competition. The mechanism of suppression may be via prevention of SRC-1 from interacting with and transactivating TR or disruption of the association between TR and TRE, preventing TR-mediated transcription (Iwasaki, et al., 2002; Miyazaki, et al., 2008; Amano, et al., 2010).

11.7 Phthalates

Since the 1930s, phthalates have been used to increase flexibility of plastics and in the production of industrial solvents, lubricants, emulsifiers, textile additives, and pesticides (Yang, et al., 2006; Chiang, et al., 2014). Additionally, phthalates, particularly high-molecular weight phthalates such as di-2-ethyl hexyl phthalate

(DEHP), are utilized as plasticizers in the production of items made of polyvinyl chloride such as consumer products, flooring, wall coverings, medical devices, and food packaging (Hurst and Waxman, 2003; Meeker, et al., 2009). Low-molecular weight phthalates like diethyl phthalate and dibutyl phthalate (DBP) have been used in personal-care products (perfumes, lotions, cosmetics and also in varnishes and coatings (Meeker, et al., 2009). More than 2 million tons of DEHP alone are produced annually around the world (Yang, et al., 2006). Due to the fact that phthalates are not covalently bound to product matrices, they can easily leach out after direct contact or heating and contaminate industrial and household products, including food packaging (Heudorf, et al., 2007; Sathyanarayana, 2008). Thus, the primary source of general population exposure to phthalates is thought to be dietary, followed by inhalation of indoor and outdoor air and dermal contact (Yang, et al., 2006; Heudorf, et al., 2007; Meeker, et al., 2009; Annamalai and Namasivayam, 2015). According to the CDC, most United States citizens have detectable concentrations of mono-ethyl phthalate (MEP), mono-(2-ethylhexyl) phthalate (MEHP), mono-butyl phthalate (MBP), and mono-benzyl phthalate (MBzP), which reflects ubiquitous exposure to these compounds. Two of the oxidative metabolites of DEHP, mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) were present at greater concentrations in the urine than those of MEHP, the hydrolytic metabolite of DEHP (Meeker, et al., 2009). Concentrations of some of these monoesters in children were higher than those seen in adolescents and adults, even when accounting for body weight (Yang, et al., 2006; Heudorf, et al., 2007; Sathyanarayana, 2008). This suggests that children may have higher exposure to phthalates than adults (Heudorf, et al., 2007). Additionally, phthalates have been identified in pregnant women, fetuses, newborns, young children, and adolescents (Schug, et al., 2011).

In the US general population, about 75% of people have detectable phthalate metabolites in urine (Kuo, et al., 2013).

11.7.1 Cell-Specific Effects

NMDRCs have been demonstrated in cell culture for DEHP, Di-n-octyl phthalate, and animal studies have verified NMDRCs in DEHP. Human health endpoints have been shown to have nonmonotonic relationships with mono-methyl phthalate, MBP, and MBzP (Vandenberg, et al., 2012).

11.7.1.1 Effects on Adipocytes and Precursors of Adipocytes

Phthalates have been positively associated with increase in body size, waist circumference, BMI, and insulin resistance in epidemiologic studies (Hatch, et al., 2008; Meeker, et al., 2009; Newbold, 2010; Tang-Peronard, et al., 2011; De Coster and van Larebeke, 2012). In analysis of NHANES 1999–2000 data (n = 4369), direct associations with BMI and waist circumference were seen in 20–59-year-old males for MBzP, MEHHP, MEOHP, MBP, and MEP. However, in women only MEP increased BMI and waist circumference in adolescent girls and 20–59-year-olds. Inverse associations for MEHP and MBzP were also noted in women (Hatch, et al., 2008; Tang-Peronard, et al., 2011; De Coster and van Larebeke, 2012; Kim and Park, 2014). NHANES also reported significant associations between concentrations of phthalate monoesters in urine (MBzP, MEHHP, MEOHP, MEP) and increased insulin resistance (Meeker, et al., 2009; Kuo, et al., 2013). A study from NHANES 2007–2010 demonstrated that high molecular weight phthalates were associated with increased obesity in male adults, and in females, increased obesity was associated with DEHP phthalates (Kuo, et al., 2013; Kim and Park, 2014). In children and adolescents, NHANES studies found that urinary levels of low molecular weight phthalates were associated with higher risk of obesity (Kim and Park, 2014).

Tissue culture studies have shown the ability of phthalates to affect adipogenic differentiation. In the initial commitment of MSCs to the adipogenic lineage, DEHP has not been found to play a role (Biemann, et al., 2012). DEHP (100 μM) has been shown to enhance adipogenesis during the hormonal induction period,

which is the stage during which activation of PPARγ directs adipogenic differentiation, mediated by C/EBPs (Biemann, et al., 2012). During this interval, DEHP increased the number of adipocytes, triglyceride content, and mRNA expression of adipocyte-specific genes, such as FABP4, PPARγ2, LPL, and adiponectin (Biemann, et al., 2012). DEHP was unable to accelerate terminal adipogenic differentiation (Biemann, et al., 2012). Adipocytes also have the ability to store MEHP, which can activate PPARγ similar to its precursor, DEHP, and induce adipogenic differentiation of 3T3-L1 preadipocytes (Hurst and Waxman, 2003; Shipley and Waxman, 2004; Desvergne, et al., 2009; Biemann, et al., 2012). The ability of MEHP to activate PPARγ may involve its ability to partially abolish the interaction between PPARγ and its corepressor NCoR, to recruit coactivator Med1, and to recruit PPARγ coactivator 1 (Casals-Casas, et al., 2008; Chiang, et al., 2014). MEHP-treated adipocytes have been further shown to decrease PPARγ expression and to increase aP2, C/EBPα, and C/EBPβ expression (Chiang, et al., 2014). In addition to DEHP and MEHP, DBP, BBP (butyl benzyl phthalate), and MBzP have also been demonstrated to activate PPARγ and to stimulate PPARγ-dependent adipogenesis in 3T3-L1 cells (Hurst and Waxman, 2003; Pereira-Fernandes, et al., 2013; Kim and Park, 2014).

In addition to PPARγ-mediated pathways, other pathways of adipogenic induction have been investigated. For example, dicyclohexyl phthalate (DCHP) has been shown to act through GR in 3T3-L1 preadipocytes to increase lipid accumulation and upregulate adipocyte protein expression during adipogenic differentiation even at DCHP concentrations as low as 100 pm (Sargis, et al., 2010). This study also demonstrated that DCHP required cotreatment with the adipogenic differentiation medium to induce adipogenesis. This indicates that phthalates may need to synergize with components of the adipogenic differentiation medium, such as insulin and corticosteroids, in order to promote adipogenic differentiation (Sargis, et al., 2010).

Effects related to diabetes and metabolism have been shown in cell culture studies. MEHP increases leptin, resistin, and adiponectin release from 3T3-L1 cells (Taxvig, et al., 2012).

Prenatal and perinatal (*in utero*) exposures of mice to DEHP have been shown to result in increased body weight of offspring and increases in visceral fat (Schmidt, et al., 2012; de Cock and van de Bor, 2014). Additionally, the DEHP-exposed female C3H/N mice also had a significant increase in body weight, food intake, and visceral adipose tissue with an increase in leptin and FABP4 mRNA expression in adipose tissue and a decrease in adiponectin (Schmidt, et al., 2012).

Figure 11.6(A) (Plate 18) shows the effects of phthalates on adipogenesis in preadipocytes and early adipocytes.

11.7.1.2 Effects on Osteoblasts and Precursors of Osteoblasts

Data from NHANES 2005–2008 (n = 398) has indicated that increasing levels of MBzP are associated with reduced total hip or femur neck BMD and osteoporosis in postmenopausal women (Min and Min, 2014).

In cell culture studies, phthalates have been shown to have either no effect, an inhibitory effect, or a cytoskeleton organization effect on osteoblasts and their precursors. A study of MC3T3-E1 cells exposed to DEHP resulted in no effect on ALP activity and cellular calcium and phosphorus contents (Kanno, et al., 2004). In another study of DEHP exposure in neonatal rat calvarial osteoblasts, a significant reduction in ALP expression and intensity and collagen synthesis. Thus, DEHP had the capability to affect differentiation of these osteoblasts and inhibit mineralization of the matrix secreted by these cells (Bhat, et al., 2013). In a study of BBP and DBP exposure, MC3T3-E1 cells and mouse primary calvarial osteoblasts were found to experience DNA base lesions. Additionally, these cells experienced an associated increase of p53, phosphorylated p53, and apoptotic proteins such as phospho-ATM, p21, and Bax levels (Sabbieti, et al., 2009). Bax translocation from the cytosol to the outer membrane of mitochondria results in cytochrome c release, which can interact with protein Apaf-1 to activate pro-caspase 9 and the caspase

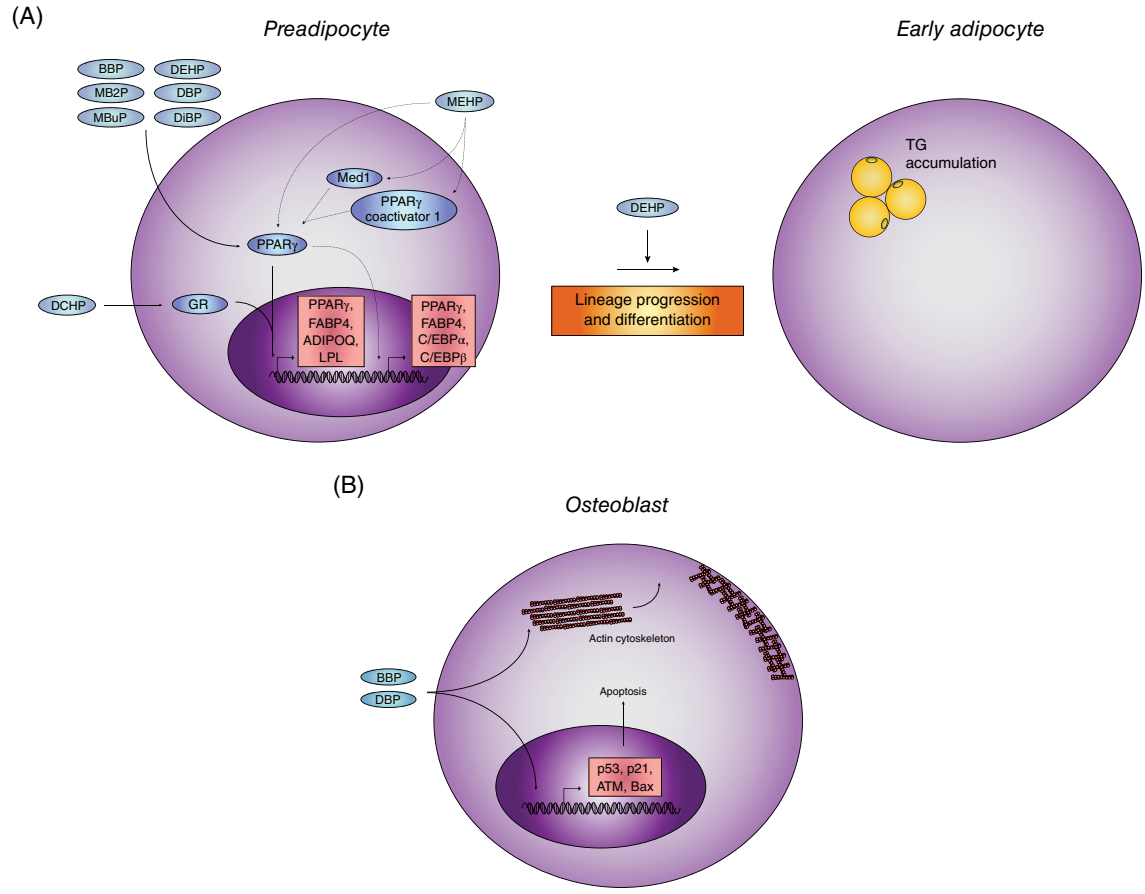

Figure 11.6 ***(Plate 18)*** *(A) The effects of phthalates on preadipocyte differentiation into adipocytes. (B) The effects of phthalates on osteoblasts. (See insert for color representation of the figure.)*

cascade (Sabbieti, et al., 2009). Thus, phthalates are capable of induction of apoptosis in osteoblasts, at least partly mediated through p53 (Sabbieti, et al., 2009). In another study of BBP and DBP exposure, Rat Py1a osteoblasts were shown to undergo reversible actin cytoskeleton alterations and cell shape change from spindle to rounded form (Marchetti, et al., 2002). Prior to treatment, the actin-based cytoskeleton was organized as parallel stress fibers throughout the cell body and its extensions, and after treatment, F-actin formed thick bundles around the plasma membrane of the spindle-shaped cells with only weak labeling in the rounded cells (Marchetti, et al., 2002). After 2 h, masses of F-actin are seen in the perinuclear region as well (Marchetti, et al., 2002). This cytoskeletal alteration has been shown to involve translational and/or posttranslational steps (Agas, et al., 2007). The cell structure of osteoblasts is essential for differentiation, activation of osteoclasts, and signal transduction of mechanical stimuli by cell-to-cell and/or cell-to-matrix contacts (Marchetti, et al., 2002).

Animal studies have demonstrated effects of several phthalates on bone. In studies of administration of DBP and di-isoheptyl phthalate to pregnant female rats, significant dose-dependent fetal skeletal malformations occurred (Ema, et al., 1994) (McKee, et al., 2006).

The effects of phthalates on osteoblasts are summarized in Figure 11.6(B) (Plate 18).

11.7.2 Molecular Effects

Phthalates have both direct and indirect estrogenic effects. DBP has been shown to have estrogenic effects mediated through ERα at 100 μM concentration (Shen, et al., 2009). Phthalates he also been shown to inhibit SULT1E1 and thus inhibit the inactivation of estrogens, resulting in an increase in levels of free active estrogens (Waring and Harris, 2005).

With regard to AR, phthalates have been shown to be antiandrogenic (Meeker, et al., 2009; Fudvoye, et al., 2014). When administered in the presence of DHT, potent antiandrogenic activity has been demonstrated by DBP, MBP, and DEHP (Shen, et al., 2009). These compounds have androgenic activity when administered alone (Shen, et al., 2009).

TR antagonist activity has been noted for DBP, MBP, and DEHP when administered with T3 (Shen, et al., 2009; Li, et al., 2014). More specifically, these phthalates may affect TH signaling by decreasing T3 binding of TR (Li, 2014).

In addition to the steroid receptor-mediated effects of phthalates, these compounds may act directly at the nuclear level. Studies have shown the ability of phthalates, particularly DEHP, MEHP, DBP, BBP, and MBP to have epigenetic effects (Singh and Li, 2012).

11.8 Areas for Future Research

While the possible roles of EDCs in diseases such as obesity, diabetes, and osteoporosis have been demonstrated in various studies, research is still lacking in several areas. In epidemiologic studies of EDC effects, future studies should further address the role of dietary factors in subjects, as exposure to many EDCs occurs primarily via ingestion. Future studies in both humans and animals should account for the total amount of adipose tissue by utilizing body fat and visceral adipose tissue as measures of obesity instead of BMI and body weight, which are not fully representative of the adiposity of a given individual.

Animal studies should be particularly careful to control for possible background levels of estrogenic chemical contamination in control animals (Vom Saal, et al., 2012). Such chemicals may be found, for example, in the food given to the animals or in polycarbonate water bottles in the case of BPA (Vom Saal, et al., 2012).

Bone marrow-derived MSCs have been demonstrated to migrate throughout the body to facilitate wound healing processes. As discussed in this chapter, bone marrow-derived MSCs and adipose tissue-derived MSCs share many features, and future research should determine whether adipose-derived MSCs have similar migratory ability. One study in particular proposed that adipogenic MSCs may migrate beyond their origin in response to stimuli such as dietary stimuli and that EDC exposure may influence this process (Janesick and Blumberg, 2011). *In vivo* studies in humans should be performed to confirm or refute this possibility.

Additionally, while epidemiologic studies have been promising, many specific mechanisms of EDCs remain to be further elucidated in *in vitro* studies and confirmed *in vivo* as discussed throughout this chapter.

Finally, the use of MSCs to help elucidate the mechanism(s) of action of EDC as developmental toxicants or differentiation altering agents holds great promise. Moreover, stem cells studies can be used to understand the relative contribution of "hormonal activity" in endocrine disrupting chemicals to the overall effect on patterns of differentiation. Studies with stem cells derived from the tissues of adult individuals are critical to understanding and predicting the effects of environmental factors on the continued health of organs and organisms that rely on rejuvenation of stem cells.

11.9 Conclusions

The effects of EDCs on human health may in part be explained by their effects on MSC differentiation capacity and biologic properties. Effects of EDCs such as DDT, BPA, certain PCBs, and phthalates to increase prevalence of obesity may be due to their ability to directly or indirectly induce adipogenesis. Additionally, because DHT-dependent AR activity inhibits adipogenesis, inhibition of DHT activity by certain organophosphate pesticides, DDT, NP, BPA, certain PCBs, and phthalates may increase adipogenesis. Effects on adiponectin production and release and also stimulation of proinflammatory cytokine section may represent diabetogenic effects of EDCs such as organophosphate pesticides, DDT, BPA, certain PCBs, and MEHP. Effects on bone formation are overall mixed and vary between EDCs. While organophosphate pesticides, alkylphenols, BPA, certain PCBs, and phthalates may have inhibitory effects on osteogenesis, DDT may have stimulatory effects. Alkylphenols may even induce apoptosis in osteoblasts. Overall, the effects of EDCs to increase adipogenesis and inhibit osteogenesis are consistent with the well-established inverse relationship between adipogenesis and osteogenesis. Further research is necessary *in vitro* and *in vivo* to clarify specific mechanisms of action in MSCs and their lineages, to determine whether general molecular effects of EDCs occur in these cells, and to relate these effects to increasing prevalence of human diseases such as obesity and diabetes.

Abbreviations

11β-HSD1	11beta-hydroxysteroid dehydrogenase 1
AChE	acetylcholinesterase
ALP	alkaline phosphatase
AP-1	activator protein 1
aP2	adipocyte protein 2
AhR	aryl hydrocarbon receptor
AR	androgen receptor
Arnt	AhR-nuclear translocator
ASC	adipose-derived MSC
BBP	butyl benzyl phthalate
BMI	body mass index
BMP	bone morphogenetic protein
BMSC	bone marrow-derived MSC
BPA	bisphenol A
BSP	bone sialoprotein
cAMP	cyclic adenosine monophosphate
Cbfa1	core-binding factor alpha 1
CBP	CREB-binding protein
CDC	Centers for Disease Control and Prevention
C/EBP	CCAAT/enhancer binding protein
CREB	cAMP response element binding
CYP	cytochrome P450
DBP	dibutyl phthalate
DCHP	dicyclohexyl phthalate
DDD	dichlorodiphenyldichloroethane or 1,1-dichloro-2,2-bis (p-chlorophenyl)-ethane

DDE	dichlorodiphenyldichloroethylene or 1,1-dichloro-2,2-bis (p-chlorophenyl)-ethylene
DDT	dichlorodiphenyltrichloroethane or 1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane
DEHP	di-2-ethyl hexyl phthalate
DHT	dihydrotestosterone
EDC	endocrine disrupting compound
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
FABP4	fatty acid binding protein 4
GLUT4	glucose transporter 4
GPR30	membrane bound estrogen GPCR
GR	glucocorticoid receptor
HEK	Human Embryonic Kidney
HGF	hepatocyte growth factor
IFNγ	interferon gamma
IGF-1	insulin-like growth factor 1
IL-1Rα	interleukin 1 receptor alpha
IL-6	interleukin 6
IL-8	interleukin 8
KGF	keratinocyte growth factor
LPL	lipoprotein lipase
MAPK	mitogen activated protein kinase
MBP	mono-butyl phthalate
MBzP	mono-benzyl phthalate
MEP	mono-ethyl phthalate
MEHHP	mono-(2-ethyl-5-hydroxylhexyl) phthalate
MEHP	mono-(2-ethylhexyl) phthalate
MEOHP	mono-(2-ethyl-5-oxohexyl) phthalate
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
NHANES	National Health and Nutrition Examination Survey
NMDRC	nonmonotonic dose-response curves
NP	nonylphenol
OC	osteocalcin
OP	octylphenol
OPN	osteopontin
OX	osterix
PDGF	platelet derived growth factor
PEPCK	phosphoenolpyruvate carboxykinase
PI-3 kinase	phosphoinositol-3 kinase
PPARγ	peroxisome proliferator-activated receptor gamma
PCB	polychlorinated biphenyl
SREBF	sterol regulatory element binding factor
SREBP	sterol regulatory element binding protein
TGF-β	transforming growth factor beta

TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor alpha
TR	thyroid hormone receptor
VEGF	vascular endothelial growth factor

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12

Epigenetic Landscape in Embryonic Stem Cells

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12.1 Introduction

Embryonic stem cells (ESCs) are usually derived from the inner cell mass (ICM) of the pre-implantation blastocyst. ESCs have the ability of indefinite self-renewal, which maintains initial stem cell pool and gives rise to multiple cell lineages. Differentiated ESCs form three embryonic germ layers: ectoderm, mesoderm, and endoderm. These germ layers are able to differentiate into different cell types and tissues of the organism. This type of developmental capacity is termed pluripotency [1]. In 1988, murine ESCs were cultured *in vitro* with the support of leukemia inhibitory factor (LIF) and irradiated mouse embryonic fibroblasts (MEFs) [2]. The cultured cells had self-renewal and differentiation potentials comparable to the ICM *in vivo*. Human embryonic stem cells were cultured *in vitro* 10 years later, which also maintained the potentials of self-renewal and pluripotency [3].

Oct4, Sox2, c-Myc, and Klf4 are essential transcriptional factors for maintaining the pluripotency state of the ESCs. It has been found that somatic cells can be induced into pluripotent stem cells (iPSCs) by these reprogramming factors [4, 5]. The iPSCs-derived mice are almost indistinguishable from those developed from the ESCs [6]. This indicated that manipulating selective transcriptional factors was sufficient to induce functional pluripotency. In addition to these transcription factors, epigenetic mechanisms have been shown to play crucial roles in the regulation of ESC state. Epigenetics provides heritable and reversible changes in gene expression through different kinds of covalent modifications [7]. Epigenetic mechanisms include DNA methylation, histone modification, ATP-dependent chromatin remodeling, and microRNAs [8–11]. Histone methylation is regulated by diverse enzymes. Methyl groups can be added to or removed by methyltransferases (HMT) and demethylases (HMD), respectively. Acetylation is regulated by acetyltransferases (HAT) and

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deacetylases (HDAC). ATP-dependent chromatin remodeling intertwines with this complex modification network, regulating chromatin structure and the accessibility to target genes. The main focus of this chapter is to summarize and discuss the epigenetic mechanisms, including DNA methylation, histone modification, and ATP-dependent chromatin remodeling, in the regulation of ESCs self-renewal and differentiation.

12.2 DNA Methylation in ESCs

DNA methylation regulates numerous biological processes in mammals and plants [12]. Three types of DNA methylation have been identified. The most well studied one is the methylation of the 5 position of cytosine pyrimidine ring within cytosine-guanine dinucleotides (CpG) [13], which results in 5-methylcytosine (5mC). The other two types of methylation are N4-methylcytosine (4mC) and N6-methyladenine (6 mA), which are found in some eukaryotes and prokaryotes [14, 15]. CpG methylation plays an important role in regulating gene expression and chromatin structure. In mammalian genomes, over 60% of CpGs are methylated, but the methylation is not evenly distributed [13]. The regions with densely clustered CpGs, termed CpG islands (CGI), are often devoid of methylation [16]. The CpG islands are usually close to gene promoters and methylation of them can lead to gene silencing. For instance, methylation of CpG islands are involved in X chromosome inactivation (XCI) and the repression of transposable elements [13, 17]. XCI is a process by which one copy of the X chromosome in female mammals is inactivated. This inactivation process is characterized by the repression of most genes on the X chromosome and transition the chromosome to a heterochromatic state labeled by CpG methylation [18]. In mammalian DNA, majority of 5-methylcytosines reside in transposons and they contribute to the repression of harmful and aggressive transposable elements [19]. Therefore, current evidence demonstrates that DNA methylation is highly correlated with gene silencing [17].

DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs) including DNMT1, DNMT3a, DNMT3b, and DNMT3-Like (DNMT3L). DNMT1 is required for the maintenance of CpG methylation status during DNA replication. Its high affinity for hemi-methylated DNA ensures the faithful copy of DNA methylation transferred from the mother strand to the daughter strand. DNMT1 is highly expressed in active cells as well as cells at the S-phase of a replication cycle [20, 21]. DNMT3a and DNMT3b catalyze *de novo* CpG methylation. DNMT3b exclusively methylates DNA repeats at the centromeric minor satellite [22]. Mutation in the C-terminal methyltransferase domain of DNMT3b has been associated with the ICF Syndrome, a disease with multiple variable symptoms such as facial anomalies and immunodeficiency [23]. DNMT3a and DNMT3b have overlapping and distinct functions in ESCs, and they are specifically targeted to unique subsets of CpG islands creating distinct DNA methylation landscapes [24]. DNMT3L does not have methyltransferase activity but can interact directly with DNMT3a/3b mediating *de novo* DNA methylation [25].

Disruption of DNA methylation is lethal to mammals. Mutation of murine DNA methyltransferases results in embryonic lethality [26]. It has been shown that DNA methylation plays an important role in ESCs differentiation and lineage commitment [27]. In the undifferentiated state, the majority of gene promoters in ESCs remain methylated, but during differentiation demethylation occurs and cell type-specific genes are expressed [28]. DNMT1 and DNMT3a/3b mutations have been used to identify the function of DNA methylation in ESCs. DNMT1 knockout reduces global DNA methylation and is lethal to ESCs development. ESCs die from autonomous apoptosis when the mutant ESCs are induced into differentiation [26]. However, DNMT3a/3b mutation behaves differently. The majority of the mutant cells remain viable but lacked the ability to initiate differentiation [22].

Studying the active DNA demethylation process further demonstrates the importance of DNA methylation in ESCs. The family of Ten Eleven Translocation (TET) enzymes oxidizes 5-methylcytosine and converts it into 5-hydroxymethylcytosine (5hmC). 5hmC has been considered as an intermediate in active DNA

demethylation process. So far only the TET family can convert 5mC to 5hmC. There are three members in the TET family: TET1, TET2, and TET3. TET1 and TET2 are highly expressed in ESCs, and knockdown of TET1 and TET2 downregulates the pluripotency-related factors [29, 30]. Because of the defects in self-renewal, the TET1/TET2-knockdown ESCs showed reduced growth and an accumulated tendency to differentiation [31]. In the mammalian genome, the balance between 5mC and 5hmC is inextricably associated with the regulation of pluripotency and lineage commitment [31].

12.3 Histone Methylation in ESCs

Histone modification is central to cell type-specific gene expression. Modification of different sites in histones often lead to distinct genomic outcomes either activating or repressing gene transcription. Histone modification usually occurs in the nucleosome. In eukaryotes, the nucleosome is formed by two copies of each histone H3, H4, H2A, and H2B, which form an octamer core wrapped around by 147 bp of DNA. The nucleosome is the fundamental building unit of the chromatin. Modification of the histones within the nucleosome can affect both nucleosome and chromatin structure. Compared to DNA methylation, histone modification is more diverse and complex. The major histone modifications include acetylation, methylation, phosphorylation, and ubiquitination [8, 32]. Histone methylation is a process by which the methyl groups are attached to lysine or arginine residues. This process is mediated by diverse methyltransferases and demethylases [33, 34]. Lysine residues can be methylated three times, which gives rise to mono-, di-, and tri-methylation (me1, me2, and me3). Different methylation statuses can lead to different functional outcomes. H3 lysine 4 tri-methylation (H3K4me3) and H3 lysine 27 tri-methylation (H3K27me3) have been shown to play an important role in ESC self-renewal and differentiation [35, 36].

H3K4 tri-methylation is mediated by at least six non-redundant complexes. These include the mixed-lineage leukemia protein 1/2 (MLL1/2), MLL3/4, and SETD1A/B complexes. A core of four subunits including DPY30, WDR5, RBBP5, and ASH2L, is shared by these complexes [37]. MLL1–4 are catalytic subunits of MLL complexes. CXXC finger protein 1 (CFP1) is unique to SETD1A/B complexes possessing a DNA-binding ability [38]. These complexes catalyze H3K4 tri-methylation at the promoters of active genes and maintain the active state of the genes. The functions of these complexes in ESCs are versatile: from global H3K4me3 deposition to more specific regulation. MLL2 null mice are Hox-gene-expression defective and embryonic lethal [39]. Depletion of MLL1 and MLL2 does not affect global H3K4 methylation but the mutant ESCs displayed cell proliferation defects due to the increased rate of apoptosis [40]. Depletion of CFP1 leads to a severe loss of H3K4 methylation at the expressed CGI-associated genes [41]. Knockdown of RBBP5 or DPY30 only has a mild effect on H3K4me3 level, but neuronal differentiation is distorted [42, 43]. Loss of WDR5 causes a dramatic decrease in global H3K4 methylation and impairment of self-renewal [44]. Therefore, H3K4 methylation is critical to ESCs self-renewal, differentiation, and development.

EZH2 (enhancer of zeste homolog 2) is the only enzyme currently known to catalyze H3K27 tri-methylation. EZH2 methylates H3K27 in the context of polycomb repressive complex 2 (PRC2). This methylation is regulated by other components of the complex such as embryonic ectoderm development (EED), suppressor of zeste 12 (SUZ12), and retinoblastoma binding proteins 4 and 7 (RBBP4/7) [45]. In ESCs, EZH2 and PRC2 act as major suppressors of differentiation-related genes. H3K27 methylation by EZH2 is required for maintaining "poised" gene silencing states before differentiation [46]. Inactivation of EZH2 and PRC2 leads to an increased tendency towards differentiation [47, 48]. Jarid2 belongs to the Jumonji (Jmj) family and is tightly associated with PRC2 complex [49]. Jarid2 is required for ESCs differentiation and development [50]. In ESCs, PRC2 components and Jarid2 are extensively co-localized, and the depletion of Jarid2 decreases the chromatin enrichment of PRC2 [51–53]. This indicates that Jarid2 is involved in targeting PRC2 to its target genes. Unlike other members of the Jmj family, Jarid2 has no histone demethylase activity [54, 55].

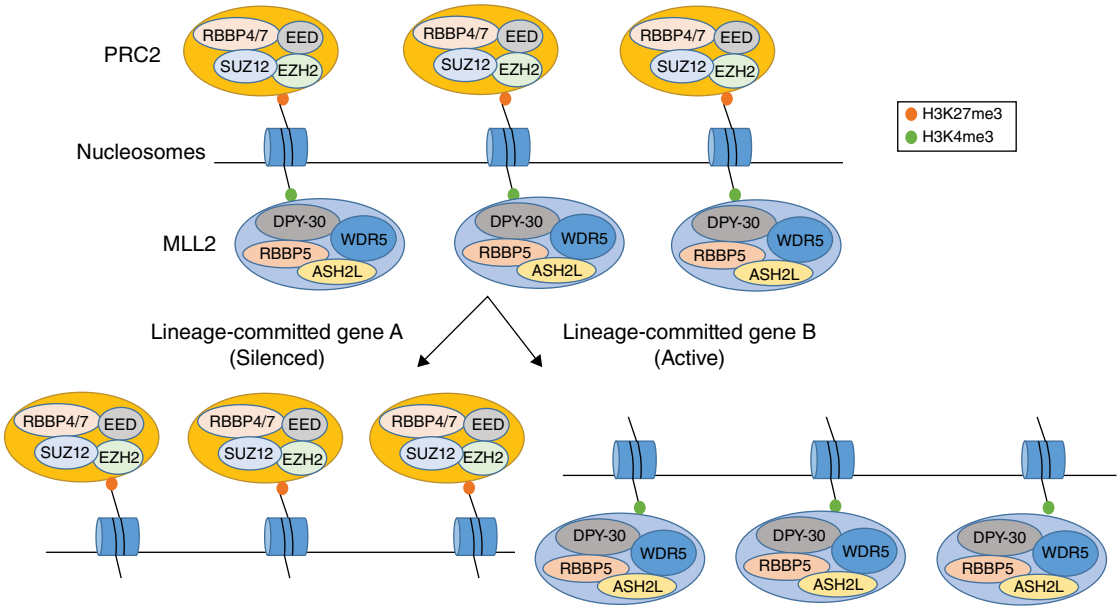

Figure 12.1 *Bivalent domain in ESCs. PRC2 and MLL2 are responsible for H3K27 and H3K4 methylation, respectively*

It has been suggested that Jarid2 might contain a nucleosome-binding domain that stabilizes PRC2 binding to chromatin [56]. It has also been reported that the methylation of Jarid2 by PRC2 triggers PRC2 activation and is required for the deposition of H3K27me3 during cell differentiation [57].

Bivalent domains are also important to ESCs pluripotency and differentiation. Such domains are characterized by the coexistence of the repressive mark H3K27me3 and active mark H3K4me3 at gene promoters (Figure 12.1) [58]. PRC2 is present at almost all bivalent domains and responsible for the deposition of H3K27me3 [59], and MLL2 is responsible for H3K4 methylation at the bivalent promoters [60, 61]. The genes in bivalent domains are usually in a silenced state but are poised for activation for differentiation. Upon differentiation, some of these domains are no longer bivalent, containing only H3K4me3 mark that is associated with active gene expression, or H3K27me3 mark which is associated with gene silencing. A small portion of promoters can maintain the bivalent state during differentiation [62]. Therefore, the balance between H3K4me3 and H3K27me3 levels is crucial for proper regulation of ESCs pluripotency and differentiation.

12.4 Chromatin Remodeling and ESCs Regulation

Chromatin remodeling is a process by which chromatin architecture is dynamically modified to allow DNA access to transcriptional factors, and thereby regulate gene expression. Four families of chromatin remodeling complexes have been shown to play roles in ESCs pluripotency including switching defective/sucrose nonfermenting (SWI/SNF), imitation switch (ISWI), chromodomain-helicase-DNA binding (CHD), and inositol TIP60-P400 complexes [63]. In mammals, SWI/SNF complexes can be separated into two subfamilies: BAF and PBAF [64]. These two complexes contain a number of shared components including the ATPase subunit BRG1 or BRM. In ESCs, loss of BRG1 led to morphology defects and reduced cell proliferation [66]. This indicates that the SWI/SNF complexes play an important role in the regulation of ESCs pluripotency and

self-renewal. BRG1 interacts with several pluripotency-maintaining transcriptional factors, such as Oct4, Sox2, and c-Myc; and they collaborate to silence the differentiation-associated genes [65]. In BAF complexes, subunits BAF250a and BAF250b are mutually exclusive, having distinct functions. Loss of BAF250a impairs self-renewal and promotes ESCs differentiation [66]; however, depletion of BAF250b leads to the delay of differentiation [67].

The ISWI chromatin remodeling family is represented by two homologs in mammals: SNF2H and SNF2L. These two homologs share a high degree of sequence identity but display different expression patterns [68]. SNF2H regulates early embryonic development, and the depletion of SNF2H is embryonic lethal and leads to the death of ICM cells [69]. The role of SNF2L in ESCs has not yet been investigated. Current data show that SNF2L regulates Foxg-dependent progenitor cell expansion in the forebrain [70], and deletion of SNF2L results in increased progenitor cell expansion and delayed neuron differentiation. SNF2L has been shown to function as part of the nucleosome remodeling factor (NURF) to promote neurite outgrowth [71].

There are nine members in the CHD chromatin remodeling family. Most of these members play an important role in ESCs [72]. CHD1 is required for the maintenance of open chromatin structures. Downregulation of CHD1 has led to heterochromatin accumulation and defects in pluripotency [74]. The loss of CHD1 can also cause self-renewal defects and decrease the expression of the pluripotency factor Oct-4 [73]. CHD3 and CHD4 are essential elements of the Nucleosome Remodeling Deacetylase (NuRD) complexes. The key component of these complexes is methyl-CpG binding protein 3 (MBD3). MBD3 regulates ESC development, and the depletion of MBD3 leads to differentiation defects [74].

The TIP60-P400 chromatin remodeling complex contains two types of nucleosome-modifying activities. TIP60 is a protein acetyltransferase that can acetylate histones H2A and H4 [75]. P400 is an ATPase which mediates exchange of H2A–H2B dimers for H2AZ–H2B dimers within nucleosomes [76]. In ESCs, the TIP60-P400 represses differentiation-related genes [78]. Knockdown of TIP60-P400 subunits leads to increased ESCs differentiation and diminished self-renewal [77, 78]. Mice homozygous for a TIP60 deletion allele die at the pre-implantation stage [79]. This indicates that the TIP60-P400 is important in maintaining ESC pluripotency.

12.5 Concluding Remarks

Epigenetics is a mechanism above genetics. It tells how, where, and when a gene is expressed. It controls gene expression without changing the underlying DNA sequence. Epigenetics is an exciting field of research that holds great promise for treatment of many diseases. The reversibility of epigenetics makes it an attractive target for therapeutic intervention. It is clear that epigenetics plays important roles in the regulation of ESC pluripotency and differentiation. The interplay among epigenetic modifications, transcription factors, and signaling pathways is necessary to resolve the fate of ESCs. More epigenetic factors have been found to participate in the regulatory network of ESCs such as H3K36me3 is involved in ESCs differentiation [80]. The acetylation of H3K18 and H3K56 and methylation of H4K20 affect DNA replication in ESCs [81]. MicroRNAs are also important players in ESCs development by regulating cell differentiation and lineage commitment [82, 83]. However, the crosstalk between these epigenetic mechanisms is still poorly understood. How a combination of multiple epigenetic modifications defines an explicit state of ESCs has not yet been well studied. Such studies remain challenging due to a large degree of epigenetic modifications and the dynamical regulation of these modifications. However, interpreting a "code" written by epigenetic mechanisms and how this code is translated into stem cell fate decision could pave the way for further understanding of human developmental biology. Such knowledge may also be valuable in stem cell-based therapies and could allow a precise control over how ESCs differentiate into specialized cell lineages.

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Part II

13

The Effect of Human Pluripotent Stem Cell Platforms on Preclinical Drug Development

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13.1 Introduction

To date, small molecule-based drugs still play a pivotal role in treating a wide range of human diseases. However, conventional preclinical drug development has faced tremendous challenges, including the cost of drug discovery, the efficacy of drugs, and the use of animal models and humanized cells. First, the cost of drug discovery has climbed considerably in the past 40 years, which makes the development of new drugs increasingly difficult for the pharmaceutical industry. Second, pharmaceutical drug efficacy and safety issues have become obstacles on the road towards personalized and precision medicine. There is a pressing need to develop highly effective drugs at reduced cost (Amiri-Kordestani and Fojo, 2012; Collier, 2009; Kola and Landis, 2004; Rawlins, 2004). Third, classical animal models used for testing preclinical drugs have fundamental species differences (compared with humans) in terms of their genomic structures and diverse signaling pathways, which have impeded their use for preclinical drug development (Eder et al., 2015; Isobe et al., 2015; Khetani et al., 2013; Rojas et al., 2015; Shih et al., 1999). Hence, there is also a need to test preclinical drugs in human models to complement current animal studies. Finally, with regard to the use of humanized cells, many primary human cells are currently employed for drug screening, and there are significant concerns regarding their variability and lack of donor diversity. Moreover, these primary cells represent a limited resource in both academic and industrial settings.

Nonetheless, human pluripotent stem cells (hPSCs) actually represent an unlimited cell resource, not only for use in regenerative medicine and cell-based therapies, but also for potential disease modeling, drug

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discovery, and preclinical drug development (Daley, 2012; Engle and Puppala, 2013; Takahashi et al., 2007; Thomson et al., 1998; Yu et al., 2007). Generally, hPSCs are classified into two major cell types: human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs). These hPSCs make possible disease modeling in a culture dish for drug discovery. As a result, these valuable cell resources are replacing the classical process for drug discovery and preclinical drug assessment. Despite rapid progress in hPSC culture *in vitro*, the application of differentiated stem cells and hPSC-based disease models to drug discovery and toxicological testing continues to face many obstacles, such as hPSC heterogeneity, differentiation immaturity, low reproducibility, and unpredictability.

All of these factors that might negatively affect drug discovery actually stem from the intrinsic properties of hPSCs as well as exogenous environmental cues that nurture these cells. The pluripotent state, one of the major intrinsic factors, determines the stemness of the cells, and also controls differentiation trajectories (Chen et al., 2014c; Dodsworth et al., 2015; Theunissen et al., 2014). Currently, we have not yet established standardized protocols for the maintenance and differentiation of hPSCs. There are simply no efficient assays or robust surface makers to monitor the exact cellular and pluripotent states of the cells. The heterogeneity and genomic instability (related to chromosomal abnormalities) of propagated hPSCs may result in mixed cell populations with altered biological properties (Baker et al., 2007; Chen et al., 2014b; Lee et al., 2013; Liang and Zhang, 2013), all of which make the interpretation of drug-screening data more difficult.

This chapter will not comprehensively review the existing literature related to hPSC-based drug discovery. Instead, it will focus on underlying insights derived from representative studies. The chapter will (1) assess core signaling pathway requirements underlying hPSC growth and directed lineage differentiation, (2) concisely analyze the basic components of *in vitro* and *ex vivo* hPSC culture, (3) discuss current hPSC platforms for low- or high-throughput (i.e., LTP or HTP) drug screening and preclinical evaluation in hPSC-based disease models, (4) explain current problems, strategies, and technical challenges through representative analyses of hPSC-based drug discovery, and finally (5) provide future considerations and suggestions for hPSC-based drug discovery.

13.2 Core Signaling Pathways Underlying hPSC Stemness and Differentiation

Typically, both hESCs and hiPSCs, which are grown both *in vitro* and *ex vivo*, possess a primed pluripotent state (Nichols and Smith, 2009; Ying et al., 2008). It is clear now that the culture of these primed hPSCs depends on signals from diverse signaling pathways. At present, only a limited number of signaling pathways have been identified that support primed hPSC growth, including basic fibroblast growth factor (FGF2), transforming growth factor β (TGFβ), and the Activin/Nodal and Noggin pathways (James et al., 2005; Vallier et al., 2005; Wang et al., 2005; Xiao et al., 2006; Xu et al., 2005). Primed hPSCs are generally passaged as small clumps and grown as colonies on feeders or on miscellaneous extracellular matrices. However, these primed hPSCs cannot grow as single cells without the support from survival factors or small molecules such as Y-27632, a potent Rho-associated kinase inhibitor (ROCKi) (Watanabe et al., 2007).

Interestingly, the primed pluripotent state of hPSCs is considerably different from that of mouse embryonic stem cells (mESCs) in a preimplantation mouse embryo, which instead exist in a naïve state (Nichols and Smith, 2009; Ying et al., 2008). Unlike hPSCs, the growth of mESCs depends on separate signaling pathways that embrace bone morphogenetic protein 4 (BMP4), signal transducer and activator of transcription 3 (Stat3), and leukemia inhibitory factor (LIF) for both survival and self-renewal (Ying et al., 2003). Using two small chemical inhibitors, (i.e., 2i) that suppress glycogen synthase kinase 3β (GSK3β) and extracellular signal-regulated kinases 1/2 (ERK1/2), with LIF, scientists have surprisingly been able to convert primed pluripotent stem cells into mESC-like naïve cells (Ying et al., 2008). Within the last 4 years,

several groups have successfully modified the 2i protocol, with an attempt to efficiently convert primed hPSCs into diverse naïve states (Chan et al., 2013; Gafni et al., 2013; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014). In general, these 2i/LIF-dependent pluripotent states share certain common transcriptome signatures, similar to those of native preimplantation epiblasts and mESCs (Chan et al., 2013; Gafni et al., 2013; Theunissen et al., 2014; Ware et al., 2014). Phenotypically, naïve hPSCs can be passaged and grown as single cells, without loss of viability after single-cell enzymatic dissociation. For example, naïve hPSCs were reported to have approximately 40% more single-cell cloning efficiency compared with primed hPSC controls (Gafni et al., 2013). Therefore, the verification of diverse pluripotent states has begun to influence hPSC expansion, genetic engineering, directed differentiation, and perhaps preclinical drug discovery.

Directed differentiation of hPSCs (with distinct pluripotent states) towards multi-lineage progenitors and adult cells represents a remarkable resource for regenerative medicine as well as for preclinical drug assessments. Directed differentiation of functionally mature cells is the ultimate goal to ensure their future application. To implement precise signaling pathways in differentiation processes is thus crucial to successful drug discovery. Accordingly, there is an increasing body of established protocols for this purpose. Understanding the basic components in these protocols is key to designing and implementing the drug-discovery pipeline.

13.3 Basic Components of *In Vitro* and *Ex Vivo* hPSC Platforms

Various culture methods for the maintenance of stemness, self-renewal, and lineage differentiation may have different effects on drug response. Intuitively, drug discovery approaches may start with hPSCs that have various pluripotent states, as described previously. Dissection of various cell culture components would facilitate the use of tailored protocols for specific purposes. Clearly, many elements can influence the quality, health, and efficacy of various hPSC cultures, which briefly include: growth medium, extracellular matrices, and environmental cues (e.g., a growth environment in a bioreactor). These components have been comprehensively discussed in a recent publication (Chen et al., 2014c). Here, only their roles in drug discovery will be discussed.

13.3.1 Growth Medium Development for Drug Discovery

The final goal for hPSC-based drug development is therapeutic use in the clinic. Thus, the formulation of any up-to-date cell culture medium should be compatible with this goal. Practically, it is better to use serum-free, xeno-free, chemically-defined medium, suitable for validating small chemical interactions with the intrinsic factors and for minimizing exogenous interference from undefined factors in the medium. In the past decade, researchers have developed many well-defined media to substitute xenogeneic components (Genbacev et al., 2005; Li et al., 2005).

KSR (**K**nock-Out **S**erum **R**eplacement) is widely used for the cell culture of hPSCs on feeder layers. TeSR1, a chemically-defined culture medium, has also been widely used to culture primed hPSCs under feeder-free conditions (Ludwig et al., 2006). However, TeSR1 can also be used for the conversion and maintenance of naïve hPSCs (Chan et al., 2013). TeSR1 medium is enriched with FGF-2 (100 ng/mL), TGFβ (100 ng/mL), lithium chloride (LiCl), γ-aminobutyric acid (GABA), and pipecolic acid (Ludwig et al., 2006). These enriched growth factors and chemicals may potentially activate or inhibit their corresponding downstream pathways or targets. Besides the well-defined FGF2 and TGFβ pathways, LiC1 interferes with the canonical Wnt-β-catenin pathway and GABA with neuronal lineage differentiation. The presence of these components may synergize or antagonize chemicals and supplements

under drug developmental conditions. For example, TeSR1 favors neuronal lineage differentiation under certain differentiation conditions (Kozhich et al., 2013), which may be associated with its pro-neural components in the medium.

Thus, researchers have made certain modifications to TeSR1. For example, E8 medium is a TeSR1 derivative that has been used to cultivate a wide-range of hESC and hiPSC lines. The E8 medium-based culture protocol relies on a non-enzymatic EDTA (ethylenediaminetetraacetic acid) passaging method, which enables tiny colonies (~5–10 cells) to plate and proliferate in a E8 medium (Chen et al., 2010; 2011). Thus, this E8-EDTA method increases hPSC culture homogeneity, improves episomal vector-based reprogramming efficiencies, and reduces experimental inconsistency. These properties are particularly advantageous for certain drug screening purposes.

13.3.2 Choices of Extracellular Components

Extracellular matrices and scaffolds, which interact with a plethora of ligands, have a clear influence on cellular and molecular behaviors of hPSCs both *in vitro* and *in vivo* (Chen et al., 2014c). Currently, Matrigel is an extracellular matrix (ECM) widely used for cell culture. This ECM is rich in collagens (types I and IV), laminin, entactin, heparan sulfate proteoglycan, matrix metalloproteinases, and some undefined growth components (Kleinman et al., 1982; 1983; Mackay et al., 1993; Vukicevic et al., 1992). Some individual components (present in Matrigel) were also found to support hPSC pluripotency and self-renewal. For example, a chemically-defined recombinant vitronectin was found to support hPSC growth *via* αVβ5 integrin (Braam et al., 2008c). Another example is the involvement of specific laminin isoforms (such as the human recombinant laminin-111, -521) in the regulation of hPSC pluripotency and differentiation (Rodin et al., 2010). Specific laminin isoforms are particularly useful for single-cell-based drug screening, without the use of the ROCKi. The drawback is the relatively higher cost of obtaining purified laminin isoforms, which might limit their use in large-scale drug screening.

In recent years, synthetic ECM materials that support the growth or differentiation of hPSCs have advanced the fields of stem cell biology and drug discovery. Synthetic ECM surfaces could mimic major signal transduction pathways of hPSCs. Usually, functional biomaterials are derived from peptide sequences of protein ligands. Properly condensed laminin peptides, high affinity cyclic arginine-glycine-aspartate (RGD) peptide, synthetic peptide–acrylate surfaces, and synthetic polymers have been used to guide stem cell growth and differentiation (Derda et al., 2007; Kolhar et al., 2010; Melkoumian et al., 2010; Villa-Diaz et al., 2010). In general, current proof-of-concept data support drug-screening strategies based on cultivating hPSCs on diverse synthetic surfaces. Hence, the generation of individual materials that are suitable for different screening purposes is a promising development for drug discovery.

13.4 Diverse hPSC Culture Platforms for Drug Discovery

Scientists have developed diverse hPSC culture platforms that have been actively used in academic research and pharmaceutic developmental sittings (Chen et al., 2014c). Briefly, these hPSC culture platforms can be classified into four categories: colony-type culture (Thomson et al., 1998), suspension culture (Amit et al., 2010; Steiner et al., 2010), non-colony type monolayer (NCM) (Chen et al., 2012), and 3D or 4D organoid co-culture (Takebe et al., 2013). High-fidelity maintenance of these hPSCs is crucial for subsequent pharmacological applications. Depending on the need, individual methods should be tailored to different purposes of drug screening assessments. Thus, large-scale maintenance of these cells could be useful, but sometimes not necessary for small to intermediate-size drug development. It is clear that each type of hPSC-based method has its pros and cons for purposes of drug discovery.

13.4.1 Colony Type Culture-Based Modules

Colony-type cultures require diverse ECM support. Human embryonic stem cell culture usually occurs on a mouse embryo fibroblast (MEF) feeder (Thomson et al., 1998). For xeno-free and clinical-grade hESC derivation, hESCs are grown on human feeders (Richards et al., 2002). In general, colony-type culture depends on conventional methods for hPSC maintenance and differentiation, which do not require the aid of small molecules for cell passaging and are compatible with hPSC growth on diverse ECMs (e.g., Matrigel, vitronectin, laminin isoforms, and synthetic surfaces) (Chen et al., 2014c). This culture method sustains diverse pluripotent states and differentiation potential, thus suitable for *de novo* hPSC line derivation, embryoid body (EB) formation, and pluripotent state conversion. Usually, hPSC colonies have low recovery rates after cryopreservation, chromosomal abnormalities, low transfection or transduction efficiency, and greater heterogeneity and variability (Braam et al., 2008b; Chen et al., 2012; 2014a; Liew et al., 2007). Thus, colony-type culture is not recommended for HTP drug-screening assays. However, it may be suitable for some differentiation experiments in which colonies-derived EBs are required. Commonly, hPSCs are expanded to generate terminally-differentiated cells through the formation of EBs.

13.4.2 Suspension Culture

Suspension culture was initially used for differentiated EB culture (Gerecht-Nir et al., 2004). Recently, this method has become commonly used for scaled-up production of undifferentiated hPSCs (Chen et al., 2014c). Suspension culture is typically initiated from the inoculation of small hPSC clumps, thus forming feeder- and matrix-free and heterogeneous EB-like aggregates. Single-cell inoculation in the presence of ROCKi may control the size of aggregates, thereby significantly improving cell yields and homogeneity (Krawetz et al., 2010; Steiner et al., 2010). In addition, suspension culture may be combined with microcarriers (abbreviated as SCMC) to adjust growth areas, reduce shear-force damage, and increase cell yields. However, the SCMC method requires coating and dissociation of different microcarriers, thus increasing variability during microcarrier attachment and cell dissociation (Fernandes et al., 2009; Lock and Tzanakakis, 2009; Oh et al., 2009). Finally, suspension culture with both microcarrier and microencapsulation (i.e., SCMC-ME) may have better protection against agitation-induced shear-force during the culture. However, it increases the complexity of hPSC expansion (Serra et al., 2011).

Collectively, suspension culture-based platforms have many advantages. They have high hPSC expansion rates, are feeder- and matrix-free, allow controllable autocrine and paracrine signaling and better monitoring of growth conditions, and are compatible with spinner flasks, bioreactors, microcarrier, and microencapsulation (Chen et al., 2014c). The disadvantages are: agitation-induced shear force, cell loss after mechanical passaging, the need of ROCKi for single-cell or small clump passaging, increased cellular heterogeneity, compromised pluripotency, and the variability of cell expansion rates. Suspension culture methods are primarily designed for hPSC expansion and cell-based therapeutics, and are not ideal for HTP drug screening. However, some benefits of suspension culture have been incorporated into micro-version bioreactors such as microfluidic bioreactors for 3D co-culture and organogenesis. More details regarding the role of 3D co-culture and organogenesis in drug discovery will be discussed in a subsequent section (Section 13.4.4).

13.4.3 Non-Colony Type Monolayer Empowers Efficient Drug Screening

Due to the significant limitations of both colony-type and suspension cultures, we and others have developed non-colony type monolayer (NCM) culture based on high-density single-cell plating (Chen et al., 2012; Kunova et al., 2013). Human pluripotent stem cells under NCM conditions remain cytogenetically normal, pluripotent, and differentiable into adult tissues of the three lineages (Chen et al., 2012; Kunova et al., 2013).

NCM culture was initially done on Matrigel in the presence of small molecules (e.g., ROCKi and JAK inhibitor I) to enhance the initial 24-hour single-cell plating efficiency (Chen et al., 2012). NCM culture greatly decreases the heterogeneity of the culture, increases hPSC expansion, and enhances the recovery rate after cryopreservation. It may be particularly suitable for conducting LTP- or HTP-type drug screening in both differentiated and undifferentiated hPSCs.

ROCKi-mediated NCM (i.e., ROCKi-NCM) may have a significant impact on gene and protein expression patterns, thus influencing differentiation trajectories. After treatment of dissociated single hPSCs with 10 μM ROCKi for 4 h, we have observed clear proteomic alterations in more than 500 regulatory proteins including redundant kinases (Chen KG et al. unpublished data). Thus, if we perform drug-screening assays using the ROCKi-NCM platform, we need to consider potential drug interactions between ROCKi and the chemical drugs of interest. To rule out the potential interference of small molecules such as ROCKi on drug-screening assays, we have also developed xeno-free and chemically defined conditions using the laminin isoform 521 (LN-521) (Chen et al., 2014a). LN-521-mediated NCM (i.e., LN521-NCM), without the use of ROCKi or JAK inhibitor I, is ideal for lineage differentiation and HTP-based drug discovery.

It has been shown that hPSCs under conventional colony-type culture are difficult to transfect or transduce (Braam et al., 2008a; 2008b; Chen et al., 2012; Liew et al., 2007). In contrast, NCM culture significantly improves the transfection efficiency of plasmids and the transduction of lentiviruses, thus empowering genetic engineering of hPSCs with shRNAs, microRNAs, oligonucleotides, plasmid DNAs, and lentiviruses (Chen et al., 2012; 2014a; Padmanabhan et al., 2012). Transient transfection or transduction allows genetic manipulation of hPSCs and subsequent functional assays within 1 week, compared with time-consuming methods that rely on isolating and characterizing individual stable clones over several months. Hence, NCM culture represents a robust tool for hPSC expansion, directed differentiation, genetic manipulation, and HTP drug screening.

13.4.4 Tissue Integration: Morphogenesis and Organogenesis

To mimic the physiological behavior of hPSCs *in vivo*, researchers have focused on hPSC-based morphogenesis and organogenesis by recapitulating the principles of developmental biology in culture dishes. By co-culture of hPSCs with certain primary cells, scientists can induce highly differentiated cells, thereby benefiting cell maturation under specific conditions. Furthermore, the maturation process of differentiated cells can be selectively augmented or inhibited by adding desired growth factors and small molecules.

Consequently, scientists have generated 3D or 4D co-culture methods to study the effects of chemical drugs on single hPSCs, single hESC-derived colonies (Villa-Diaz et al., 2009), and signaling trajectories among individual hPSCs and different types of adult cells (Moledina et al., 2012; van der Meer et al., 2013). Microfluidic bioreactors, micro-scale versions of conventional bioreactors, provide a highly manageable 3D niche for hPSCs. Micro-environmental cues are spatially orientated within cells and extracellular matrices, and controlled by the concentration gradients of growth factors, nutrients, gases (e.g., oxygen and carbon dioxide), and temperature (Cimetta et al., 2009).

Takebe and colleagues have established an efficient 3D-coculture protocol for directed differentiation of hiPSCs toward hepatic endodermal cells (hiPSC-HEs), which are capable of self-organizing into functional 3D liver buds (Takebe et al., 2013). These liver buds are able to metabolize drugs, thus providing a useful model to test hepatotoxicity of chemical drugs. In addition, complicated neuronal organoids might pave the way to the study of sophisticated neuronal disorders in multi-dimensional culture systems. To generate neuroectodermal organoids using 3D hPSC co-culture, several groups have employed cellular structure dynamic patterning and self-organization to derive complicated tissue patterns, including cortical neurons, the 3D optic cup structure of the neural retina, and various brain regions containing progenitors (Eiraku et al., 2008; Lancaster et al., 2013; Nakano et al., 2012). Collectively, these complicated neuronal structures are now called “cerebral organoids.” Clearly, microfluidic bioreactors combined with tangible tissue engineering and

micro-organoids may have widespread applications in drug discovery and preclinical drug development. Spatiotemporal control of intercellular interactions in this system would reveal potential precision drug-interaction patterns and predictable outcomes, which are essential to assess preclinical drugs *in vitro*.

13.5 Representative Analyses of hPSC-Based Drug Discovery

To better understand the role of hPSCs in drug discovery, analysis of representative studies in three-lineage differentiation will now be presented, particularly focusing on neuroectodermal disease models that shed light on progressive and fatal neuronal diseases, hPSC-derived hepatocytes directly involved in drug metabolisms, and cardiomyocytes that are used for toxicological assessment. This analysis is based on the feasibility of technological platforms, the strength and weakness of each study, and their potential applications in future drug development.

13.5.1 Neuroectodermal Disease Models for Drug Assessment

Currently, researchers are attempting to develop effective drugs for progressive and fatal neuronal diseases such as amyotrophic lateral sclerosis (ALS). So far, no effective therapies exist for ALS, the most common form of progressive neuromuscular diseases. In ALS patients, both the upper and the lower motor neurons undergo progressive degeneration and/or cell death, resulting in dysfunctional neurons that are incapable of transmitting signals to muscles. The muscles gradually deteriorate, atrophy, and show twitching symptoms (or fasciculation). Ultimately, the brain loses control of the voluntary movement of skeletal muscles.

In 1995, a clinical trial was conducted to treat ALS patients with riluzole. However, it only prolonged patients' survival for several months, mainly those with difficulty in swallowing. Moreover, riluzole does not repair previous damage to motor neurons. Although the mechanism of action of this drug is not clear, it may protect motor neurons by reducing the release of glutamate (www.ninds.nih.gov). Seventeen years later, a randomized, double-blind, Phase III clinical trial of dexpramipexole, a new drug for ALS, was completed in 2012. Based on the combined assessment of function and survival (CAFS) score, least-square mean CAFS scores at 12 months did not change significantly between participants in the dexpramipexole group and those in the placebo group (P value = 0.86) (Cudkowicz et al., 2013). The next year, a phase II/III trial combined olesoxime, another new drug for ALS, with riluzole, but this combination provided no significant beneficial effect in ALS patients (Lenglet et al., 2014). Thus, the preclinical drug developmental efforts exerted for ALS patients represent unsuccessful attempts at drug discovery over the past twenty years. Recently, a small molecule survival screen of motor neurons derived from both wild-type and mutant SOD1 mESCs led to the identification of kenpaullone, a dual inhibitor of both GSK-3 and HGK kinases (Yang et al., 2013). Kenpaullone improves ALS-hiPSC-derived motor neurons, and is more active than either olesoxime or dexpramipexole (Yang et al., 2013). Thus, kenpaullone has become a potential candidate for treating ALS, providing clear evidence that hPSC-based disease models and drug screening hold promise for treatment of ALS patients.

To demonstrate the utility of hPSC-based disease modeling in drug-mediated phenotype-rescue experiments, Dr. Alysson Muotri and colleagues (University of California, San Diego) produced hiPSCs from Aicardi-Goutieres syndrome (AGS) patients with mutations on the *TREX1* gene and compared them with isogenic controls (Brennand et al., 2015). TREX1 (Three Prime Repair Exonuclease 1) is a major DNA exonuclease that digests mismatched single-stranded DNAs at 3' and DNA fragments from endogenous retroelements (e.g., L1, LTR, and SINE elements). Mutations in this gene cause Aicardi-Goutieres syndrome, chilblain lupus, Cree encephalitis, and other autoimmunity diseases. Interestingly, Muotri and colleagues found that TREX1-mutant cells were unable to clear single-stranded DNA (ssDNA) at the cytoplasm and had decreased expression of neuronal markers. Fortunately, the phenotype could be reversed by anti-HIV drugs

(Brennand et al., 2015). A mouse model of TREX1 mutations could not recapitulate the neurological phenotype of AGS, thus indicating the importance of humanized cell sources (such as hiPSC-derived neurons) for drug discovery of AGS inhibitors.

13.5.2 Hepatic Models for Drug Assessment

The endoderm, one of the three germ layers in early human embryos, generates the gastrointestinal and respiratory tracts, the urinary and auditory systems, and endocrine glands and organs. The liver, a vital organ derived from the gastrointestinal tract, has many important physiological functions. It processes, stores, and metabolizes absorbed nutrients, detoxifies various metabolites, and decomposes alcohol and many drugs. Hence, the pivotal role in drug metabolism of the liver makes its cells (i.e., hepatocytes) an important resource for studying toxicology and for drug discovery. To demonstrate the utility of co-culture systems for modulating hepatic maturation, Deleve and colleagues co-cultivated rat hepatic stellate cells with freshly isolated sinusoidal endothelial cells, which inhibited activation of stellate cells (Deleve et al., 2008). Quiescent hepatic stellate cells (beneath the sinusoidal endothelial cells) are adjacent to the hepatocytes. When activated, hepatic stellate cells produce collagen, which may be a major cellular mechanism that causes liver fibrosis.

Physiological properties of organotypic 2D and 3D co-culture models using primary hepatocytes and alternative cell resources have essentially overcome some of the challenges concerning toxicity and thus are suitable for drug development (Godoy et al., 2013; Khetani and Bhatia, 2008; LeCluyse et al., 2012). However, due to the lack of consistent quantity and quality of primary cells, hepatocyte resources may be limited for large-scale drug discovery. Obviously, it is important to have advanced culture systems that support stable hepatocytes and non-parenchymal cells. The culture system would be expected to sustain essential hepatic physiological properties (e.g., drug metabolism and transport) under prolonged culture conditions.

Hepatocyte-like cells derived from both hESCs and hiPSCs have been 3D co-cultured with Swiss 3T3 cell sheets, which synthesize type I collagen. This co-culture system facilitates hepatic maturation, as indicated by expression of hepatocyte-related markers (e.g., cytochrome P450 and conjugating enzymes) and secretion of albumin (Nagamoto et al., 2012). Generation of hepatocyte-like cells from hiPSCs was achieved by co-culturing EB cells with liver non-parenchymal cell line TWNT-1 (Javed et al., 2014). Functional maturity of hiPSC-derived hepatocytes was induced via intercellular interactions with 3T3-J2 MEFs in micropatterned co-culture (Javed et al., 2014). Thus, optimization of cellular components of the hepatic co-culture system should enable the maintenance of vital hepatocytes for future drug assays.

Furthermore, hiPSCs-derived hepatocytes (iHEPs) under micropatterned co-cultures were used in LTP screening of 47 drugs in 96-well plate for 6 days. Multiple functional parameters (e.g., ATP, albumin, and urea) were assayed to evaluate hepatocyte toxicity (Ware et al., 2015). The researchers found that this method had 65% sensitivity for classifying hepatotoxic drugs and 100% specificity for defining 10 non-toxic drugs. Thus, these data suggest iHEP co-culture methods could be a robust tool for screening hepatoxic drugs and potentially used for screening of therapeutic drugs in iHEP-derived disease models.

13.5.3 Cardiomyocytes for Cancer Drug Discovery

Mesodermal differentiation produces a wide range of progenitor cells that subsequently form connective tissue, cartilage, bone, fat tissue, cardiac and skeletal muscles, and blood cells of all sublineages. Among mesodermally-differentiated cells, cardiomyocytes and bone marrow cells are frequent cytotoxic-drug targets in cancer patients. Thus, cardiotoxicity and myelotoxicity (i.e., bone marrow suppression) are common complications after chemotherapy. With regard to these issues, hiPSC-derived mesodermal tissues open vital avenues to understanding and predicting potential organ toxicity in individual cancer patients, which could significantly lower the cost of cancer clinical trials. Cardiotoxicity represents one of the major causes that leads to

the withdrawal of preclinical drugs. In the following sections, drug-induced cardiotoxicity will be discussed as an example of the role of hPSC-derived cellular models in preclinical drug development, particularly for protein kinase inhibitors.

Specific inhibition of the activity of protein kinases that promote cancer growth and metastasis has transformed cancer therapies and enhanced survival rates in many cancer patients. These small chemical inhibitors that target kinases have facilitated drug expansion for cancer therapy (Force and Kolaja, 2011). However, they have also produced substantial off-target toxicities, which include cardiotoxicity (Chu et al., 2007). To date, there are approximately 11 kinase inhibitors on the US market. Definite clinical cardiotoxicity was observed in sunitinib (Sutent, Pfizer, 2006), a proven inhibitor with multiple targets (e.g., VEGFR, PDGFR, FLT3, and KIT) used to treat renal cell carcinoma and gastrointestinal stromal tumor. Approximately, 18% of patients with gastrointestinal stromal tumors developed congestive heart failure (CHF) due to a significant decrease in left ventricular ejection (Chu et al., 2007).

Early hPSC-derived cardiac cells were a heterogeneous population containing atrial, ventricular, and nodal-like cells, with the beating phenotype only appearing in mature cardiomyocytes, incompletely developed electrophysiological properties (similar to embryonic cardiomyocytes), immature sarcomeric structures, and deficiency in a prominent rectifier potassium current (I_{Kr}) in hPSC-derived cardiomyocytes (Braam et al., 2009; Kehat et al., 2004; Satin et al., 2008; Zhang et al., 2009). Thus, these immature cells may not be suitable for large-scale drug screening purposes. Nevertheless, using patch clamping and multiple electrodes, researchers have shown that several cardioactive molecules (such as verapamil, terfenadine, cisapride, E-4031, sotalol, and quinidine) display expected changes (Braam et al., 2010; Liang et al., 2010). These data demonstrate the feasibility of using hPSC-derived cells for drug discovery. However, functional mature cells should be developed and used in a physiologically compatible system in order to be successful.

Encouragingly, current data suggest that hPSC-derived cardiomyocytes could be used for studying chemical drug-related cardiotoxicity. Recently, Hinson and coworkers successfully applied 3D cardiac micro-tissues engineered from hiPSCs to model dilated cardiomyopathy, a main cause of heart failure and premature death, and to assess the pathogenicity of titin gene variants related to this disease (Hinson et al., 2015). They identified titin pathogenic mutations that disrupt sarcomere structures that control contractile properties and response to mechanic stress and β-adrenergic signaling (Hinson et al., 2015). Mathur et al. reported a cardiac micro-physiological system (MPS) based on hiPSC-derived cardiac tissues, a useful platform to predict cardiotoxicity in vitro (Mathur et al., 2015). The MPS is able to perform biological, electro-physiological and physiological analyses on physiologically relevant tissue with predictable vasculature perfusion and simulated velocity profile in the MPS. Notably, the MPS supports confocal microscopy imaging of multiple-layer thick structures. In addition, the MPS allows us to maintain hPSC-derived tissues over several weeks (Mathur et al., 2015). So far, the MPS has been used to test several classes of cardiac drugs, including β-adrenergic agonist (e.g., isoproterenol), β-adrenergic antagonist (e.g., metoprolol), hERG channel blockers (e.g., E-4031), and calcium channel blockers (e.g., verapamil). Key pharmacological values such as half maximal inhibitory/effective concentration values (IC_{50}/EC_{50}) from the cardiac MPS are consistent with the data on tissue scale references (Mathur et al., 2015). The integrative potential of the MPS with other microfluidic-based micro-organoids makes the MPS a powerful tool, suitable for a wide range of pharmaceutical applications and preclinical drug development.

13.6 Current Challenges and Future Considerations

Human pluripotent stem cells and their differentiated cells, spotlighted in a drug-discovery era, undoubtedly have many unresolved issues. As a cell-based tool, the maturity or functionality of differentiated cells is obviously the most important subject. Only with the desired cell sources would we be able to address subsequent

challenges in this drug-discovery era. The leading challenges include the complexity of drug discovery (associated with both genetic and epigenetic factors) as well as many other notable tasks such as data reproducibility and variability.

13.6.1 Dimensionality, Maturity, and Functionality of Differentiated Cells

In general, current hPSC culture platforms can be implemented in multi-dimensional modes (i.e., in 2D, 3D, and 4D environmental cues) to meet a specific demand. Conditions that mimic *in vivo* microenvironments, which could be achieved in micro-bioreactors, include cell density, controllable temperature, humidity, osmosity, acidity, rigidity of ECMs, gas diffusion rates, and oxygen concentrations. Under these conditions, autocrine, paracrine, and telecrine signaling could be optimized by providing exogenous growth factors, ligands, and permeable small molecules. Thus, NCM platforms can be used for single-cell kinetic and lineage analyses. Multi-dimensional organoids can be employed to study drug effects on complex cellular behaviors.

Despite the availability of multi-dimensional hPSC platforms, hPSC-derived cellular structures still cannot faithfully represent human tissues *in vivo*. Some physiological barriers (e.g., blood-brain barriers), which play important roles in drug uptake and export, cannot be easily reconstructed. Frequently, organized cellular structures are similar to premature embryonic or neonatal tissues. In the case of cardiac tissues, some physical properties, such as the low stiffness of cardiac tissue, may be associated with the low efficiency of cardiac beating rates. It seems difficult to couple the engineered tissues to host cellular structures. Therefore, hPSC-derived tissues may not recapitulate the impact of epigenetics, somatic factors, and distant immunomodulatory effects on tissue responsiveness to small molecules and peptides.

To overcome these barriers, it is important to fully characterize the derived tissues using native tissues as controls (standards) prior to any major drug discovery projects. Here, hepatic differentiation can be used as an example to illustrate certain strategies that may lead to the resolution of various problems. First, we should be aware of the optimal maturity and functionality of differentiated cells *in vitro* when planning a drug discovery project. With regard to the functionality of hepatic architectures in a co-culture system, a physiologically relevant cell ratio (i.e., 80% hepatocytes to 20% non-parenchymal cells) should be included in the system. Second, to monitor hepatic maturity, we should examine a panel of critical regulators and enzymatic drug metabolizers. Core hepatic regulators are orphan nuclear receptor (PXR), hepatocyte nuclear factor 4α (HNF4α), bile acid receptor (FXR), oxysterol receptor LXR-α (LXR), and vitamin D3 receptor (VDR) in cultured cells. One defective core regulator might result in altered expression of hundreds of downstream genes. Critical enzymatic drug metabolizers consist of cytochrome P450 family members (CYPs). CYPs are the predominant enzymes that control the metabolism of the majority of drugs in humans, resulting either their inactivated or activated compounds (Guengerich, 2008). Changes in CYP protein expression or enzymatic activities affect the metabolism and clearance of various chemical drugs. Thus, we need to examine the protein levels of CYPs and determine their enzymatic activities in hPSC-derived hepatocytes.

Furthermore, we need to examine the presence or absence of important hepatic drug transporters (e.g., ABCA1, ABCB1, ABCB4, ABCB11, ABCC2, ABCC7, ABCG2, ABCG5, and ABCG8) (Wlcek and Stieger, 2014). ATP-binding cassette (ABC) multidrug transporters play a fundamental role in the distribution and excretion of chemical drugs in a broad range of tissues and are basic components of the blood-brain, blood-testicular, and blood-placental barriers (Chen and Sikic, 2012; Dean and Annilo, 2005; Deeley et al., 2006; Gottesman et al., 2002). Moreover, ABC transporters are involved in intrinsic and acquired cancer drug resistance and in many metabolic and genetic diseases. Despite the enthusiasm concerning novel strategies of drug discovery through HTP screening, some of the effective kinase inhibitors that have been developed or are being established are likely to be effluxed from cancer cells by ABC transporters. For example, the potent

tyrosine kinase inhibitor STI571 (also known as imatinib mesylate) used for treating many cancers is actually a substrate of both ABCB1 and ABCG2, which may account for clinical drug resistance to STI571 and subsequent treatment failure (Burger and Nooter, 2004; Burger et al., 2004). Thus, multidrug transporters including ABCs have an important impact on various stages of the drug-discovery pipeline. Accordingly, hPSC-derived tissues such as hepatocytes that are used for drug discovery purposes should be routinely tested for their capacity to bind, accumulate, and export drugs. Moreover, these assay results should be compared with those of wild-type hepatocytes and mutant hepatocytes that harbor mutations at drug-binding sites of drug transporters (Ambudkar et al., 1999; Chen et al., 2000; Loo and Clarke, 2005). Understanding the effect of transporters could help to accurately measure the functional maturity of engineered tissues *in vitro* and evaluate their potential impact on preclinical drug development.

13.6.2 Complexity: Genetics versus Epigenetics

Resolving the complexity of the biological system in humans is an essential task in drug discovery. Hence, the emergence of hiPSC-based remodeling of the complexity of the brain is an initial step. The combined use of genome-editing systems such as CRISPR/Cas, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) can facilitate the creation of high-fidelity hiPSC clones or isogenic lines that contain various mutations to study reverse and forward genetics in a dish. Obviously, these approaches can be integrated for HTP drug screening in 384- and 1536-well plate formats.

Somatic mosaicism, which depicts the existence of genomic structural or sequence variations from cell to cell within each individual, is an emerging topic in the field of stem cell biology (Brennand et al., 2015). To extend this concept to hPSCs, genomic instability-associated chromosomal abnormalities, a frequently encountered phenotype in stem cell culture, can produce a cell population with a different chromosomal makeup, likely resulting in chromosomal mosaicism in hPSC-derived tissues as well. Thus, somatic mosaicism or chromosomal mosaicism may have the potential to increase the variability of datasets and decrease reproducibility of drug-screening experiments. Likewise, epigenetic or somatic factors as well as other distant immunomodulatory factors may not be recapitulated in our *in vitro* systems, regardless of 3D autocrine and paracrine signaling simulation. The absence of these epigenetic factors may potentially modify the properties of hPSC-derived tissues, contributing to discrepancies in cell or tissue response to small molecules and peptide ligands.

13.6.3 Other Notable Factors

Briefly, other major problems are data reproducibility and variability, platform scalability and compatibility, and certain economic considerations. To reduce experimental variability and increase data reproducibility, we need to precisely define stem cell states and their tentative fates. For example, mESCs exist in the naïve pluripotent state, which does not reflect the primed state in hPSCs. Thus, drug-screening data from mESC-derived tissues should be validated in both naïve and primed hPSCs. It is necessary to determine definitive surface markers, robust reporters, and informative assays for each specific cellular state. Multi-laboratory collaborative efforts including protocol optimization, standardization, tissue integration, and perhaps differentiation automation could facilitate comparative studies between academic and pharmaceutical researchers. In terms of the scalability, compatibility, and cost, we ought to determine which drug-screening projects and mechanisms can be studied in a specific hPSC-based model. We also need to choose between LTP- and HTP-type drug screenings. For engineered cellular models, once we have appropriately characterized and validated them, we could simplify and standardize all procedures, thus considerably decreasing the cost of finding new drugs. No doubt, unraveling these prospective problems could be challenging.

13.7 Concluding Remarks

In summary, an analysis of current hPSC culture and differentiation platforms and strategies to improve currently decreased productivity of preclinical drug development has been presented. It is time to re-evaluate the drug development process, embrace new technological innovation in the post-genomic era, and more importantly to achieve a full understanding of pharmacogenomics, which affects therapeutic efficacy in population-based and individualized medicine. Numerous hPSC platforms can be used for drug discovery and preclinical assessments. Certain novel hPSC culture platforms appear to be robust and scalable, making them powerful tools for drug discovery.

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14

Generation and Application of 3D Culture Systems in Human Drug Discovery and Medicine

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14.1 Introduction

From fertilization until birth, the embryo undergoes a series of dramatic changes. This culminates in the formation of specialized cell types and tissues, essential to normal organ function. In order to achieve such a level of sophistication, tissue organization and synchrony are key. At the single cell level, increasing specialization takes place through a process known as cytodifferentiation, resulting in specialized tissue formation.

Organogenesis is a complex process with cell-cell interactions at the molecular and mechanical level being key to their formation (Sasai et al., 2012). Furthermore, cells communicate with their environment through nanoscale cues provided by surrounding extracellular matrix (ECM) (Stevens and George, 2005). In fact, the specificity and homeostasis of the tissue is maintained through interaction of cells with neighbouring cells and ECM (Kleinman et al., 2003). Historically, tissue engineering has employed primary mammalian cells and cell lines to study biology in a dish or *in vivo*. With the advent of mouse and human pluripotent stem cells (Thomson et al., 1998) research has focussed on developing renewable sources of human somatic cells to build tissue (Szkolnicka et al., 2013). Researchers have used basic developmental biology principals *in vitro* with hope to create cells and microtissues to model human biology 'in a dish' and the potential applications in regenerative medicine and drug discovery (Reubinoff et al., 2000, Keller, 2005, Mummery et al., 2007, Hay et al., 2008a, Hay et al., 2008b, Medine et al., 2013, Godoy et al., 2015, Villarin et al., 2015, Szkolnicka et al., 2016).

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Traditionally, culturing cells in two-dimensional (2D) monolayer has been used predominantly due to ease. However, the conditions that cells experience in 2D monolayer cultures is far from their natural microenvironment, as tissue-specific architecture, cell-cell communication and mechanical and biochemical cues are lost under such simplified culture conditions (Pampaloni et al., 2007). Culturing cells on a flat substrate such as conventional cell culture plastic results in remodelling of the cytoskeleton, leading to cell flattening and alterations in nuclear shape (Thomas et al., 2002). This manifests in dramatic changes in chromatin structure (Vergani et al., 2004), which subsequently alter gene expression and protein synthesis. Due to limited cell interactions, it was suggested that culturing cells on rigid surfaces encouraged proliferation and inhibited differentiation (Cukierman et al., 2002). Therefore, developing new systems to culture cells in more physiologically relevant environment is highly desirable and could advance our understanding of tissue morphogenesis (Yamada and Cukierman, 2007), providing better *in vitro* models (Bhadriraju and Chen, 2002). Numerous approaches to develop suitable conditions for culturing cells in three-dimensional (3D) cell configurations have been developed leading to the birth of tissue engineering.

Tissue engineering is a multidisciplinary field of science that includes; biology, chemistry, physics and engineering with the goal to build and restore function by mimicking native tissue (Langer and Vacanti, 1993, Griffith and Naughton, 2002, Langer and Tirrell, 2004). In the quest for developing 3D tissue, the field of tissue engineering has expanded in past two decades. Several polymers have been tested, various type of bioreactors have been manufactured and the researchers have come a long way; However, *in vitro* fabrication of 3D tissue has remained a challenging pursuit with traditional tissue engineering based on seeding cells on preformed scaffolds. These attempts have been enabling, however, they do suffer significant limitations. This has led to novel approaches that include scaffold free 3D culture and tissue bioprinting (Berthiaume et al., 2011, Lanza et al., 2011).

14.2 Traditional Scaffold-Based Tissue Engineering

Traditional tissue engineering approaches compose of two distinct steps, the fabrication of the scaffold followed by cell seeding. Scaffolds provide suitable matrix for cell attachment, migration, proliferation and differentiation. In addition, scaffolds provide structural support to maintain the shape and mechanical properties of the target tissue. Due to importance of the scaffold, extensive research has been performed to identify new materials and new fabrication methodologies to improve the tissue performance.

14.2.1 Materials for Fabrication of Scaffolds

Key requirements for a tissue engineering scaffold include biocompatibility, biodegradability, suitable geometry and mechanical properties. As one of the main components, extensive effort has been put into development of new materials to manufacture scaffolds. As a result, a wide range of materials and several manufacturing procedures are available to researchers to choose based on tissue of target.

Polymeric biomaterials can be classified into various categories that include, natural polymers, synthetic biodegradable and non-biodegradable polymers, hydrogels and ceramics. This is usually based on their chemical and structural compositions and biological characteristics.

14.2.1.1 Naturally Occurring Polymers

Polysaccharides, such as glycosamino glycans, alginates, agarose, chitosan and proteins, including collagen, laminin, fibrin, fibronectin, elastin, gelatin and silk are examples of naturally occurring polymers that are

widely used in tissue engineering. Better interaction, improved performance and minimal inflammatory response at site of implantation can be achieved using natural materials (Mano et al., 2007).

Since proteins are the major components of extracellular matrix, a large body of research on natural polymers has focused on potential applications of natural and genetically engineered proteins such as collagen, fibronectin, laminin, elastin and vitronectin in regenerative medicine and tissue engineering. Collagens are a large family of triple helical proteins which are the most abundant protein in the mammalian body, accounting for around one-third of total body protein content (Shoulders and Raines, 2009). As the main component of connective tissue, collagens have attracted a great deal of interest and their potential use has been studied for a wide range of applications including bone (Lyons et al., 2010), vascular (Park et al., 2009) and soft tissue repairs (Pabbruwe et al., 2010). Fibronectin is another ECM protein, which is a multi-domain glycoprotein and mediates its wide range of biological functions through interaction with cell adhesion receptor integrins (Pankov and Yamada, 2002). Therefore, fibronection as whole or its binding sequences, such as RGD, have been used to improve cell adhesion and proliferation of other polymeric scaffolds such as alginate (Rowley and Mooney, 2002) and chitosan (Amaral et al., 2009). Since ECM has a complex mixture of macromolecules with tissue-specific biological and biophysical characteristics, protocols have been developed to decellularize tissue ECM to study ECM-stem cell interactions *in vitro* and have potential applications in regenerative medicine (Gattazzo et al., 2014).

14.2.1.2 Biodegradable Synthetic Polymers

These type of polymers are synthetic and their properties can be tailored. Biodegradable polymers mainly degraded through hydrolysis. The most extensively used biodegradable polymers are poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their co-polymers poly(lactide-co-glycolide) polyethylene glycol (PEG), polycaprolactone (PCL) and polyurethane (Doppalapudi et al., 2014).

14.2.1.3 Bioactive Glass and Glass Ceramics

Bioactive glass and glass ceramics are manufactured by melting or sintering inorganic raw materials to produce amorphous or crystalline solid body and typically characterized by very low elasticity, high mechanical stiffness and a hard brittle surface. Due to their mechanical properties bioceramics including hydroxyapatite, calcium phosphates, tricalcium phosphates and silicate and borate bioactive glasses have been used for bone tissue engineering. Based on resorbability, bioceramics can be classified as biodegradable or resorbable (non-inert), bioactive or surface active (semi-inert) and non-resorbable (relatively inert) (Gerhardt and Boccaccini, 2010, Rahaman et al., 2011).

14.2.1.4 Hydrogels

Hydrogels are hydrophilic polymer chains that can be cross-linked to form 3D structures. Hydrogels are the most commonly used materials for fabrication of complex 3D structures due to their unique characteristics including optical clarity, tuneable mechanic property and degradability, non-immunogenicity, high compatibility and high water content. Since pioneering work on cross-linked poly(hydroxyethylmethylacrylic)acid [poly(HEMA)] (Wichterle and Lim, 1960), several natural, semi-synthetic and synthetic hydrogels have been designed for stem cell and cancer research, immunomodulation, *in vitro* diagnosis, drug delivery and tissue engineering applications. Natural hydrogels such as alginate, collagen, hyaluronic acid, fibrin and ECM fall into the naturally occurring polymers category as described previously. Synthetic hydrogels such as poly(ethylene glycol) (PEG) and methyl acrylate derivatives have been used widely for various applications in stem cell research including photolithography and microfluidics. To improve cell-compatibility,

some of the hydrogels can be covalently conjugated with ECM proteins such as fibrinogen, albumin or collagen to make semi-synthetic hydrogels (Seliktar, 2012). Conjugation has been achieved through a range of reactions including step-wise copolymerization of hydrophilic polymers with protein or polysaccharides, disulfide bonding, photo-initiated free-radical copolymerization, metal-free Click chemistry, such as Michael addition, and Schiff-bass formation reactions (Hennink and van Nostrum, 2002).

14.2.2 Fabrication Methods

Various fabrication methods have been developed for manufacturing of scaffolds from natural or synthetic polymers. The fabrication techniques can be categorized into conventional and advanced techniques. Fabricated scaffolds using a conventional method such as freeze-drying, melt moulding, gas foaming and phase separation has a sponge-like structure with uniformly distributed pores that lacks interconnectivity and regularity in shape. On the other hand, geometric characteristics (pore size, distribution and interconnectivity) of scaffolds fabricated using advanced methods including stereolithography, direct-write electrospinning and 3D-printing can be controlled. In addition, several fabrication techniques have been developed to create microscale hydrogels for tissue engineering applications. In this section, the essential basic of the most common fabrication methods to create advanced scaffolds and microengineered hydrogels will be provided.

14.2.2.1 Photolithography

Incorporation of micro- and nano-scale topographic features was developed by adaptation of a technique routinely used in the production of semiconductors by the electronics industry (Curtis and Wilkinson, 1997, Rashidi et al., 2014). In photolithography, a photomask is used to incorporate desired pattern on hydrogel precursors, which undergo a crosslinking reaction with photoinitiator when exposed to ultraviolet (UV) light. Photolithography is a low-cost and simple hydrogel fabrication technique with a resolution in range of millimetres to micrometres (Khademhosseini and Langer, 2007, Slaughter et al., 2009). Modified techniques such as electron beam lithography can be used to create sub-10 nm topographic features (Chen and Ahmed, 1993, Vieu et al., 2000). The major disadvantages of these techniques are potential DNA damage due to formation of free radicals from photoinitiator following exposure to the UV (Slaughter et al., 2009) and the requirement of photocurable hydrogel precursors (Khademhosseini and Langer, 2007).

14.2.2.2 Soft Lithography

Soft lithography is a set of techniques to create microscale patterns on hydrogels using elastomeric stamps (Whitesides et al., 2001). Soft lithography offers a number of exclusive features for biological applications including the ability to control the molecular structure of surfaces and to pattern the complex biological molecules with controlled topography and spatial distribution (Whitesides et al., 2001, Khademhosseini et al., 2007).

14.2.2.3 Microfluidics

Microfluidics is a term that has been used for the science and technology of manipulation of small (10^{-9}–10^{-18} litres) volume of fluids in channels with dimensions of tens to hundreds of micrometres (Whitesides, 2006). Although the primary application of microfluidic technology was in molecular analysis, it has been adapted for various biological applications in the past two decades, of which some of these applications will be discussed in more detail in following sections.

14.2.2.4 Emulsification

Emulsification process is a technique to form hydrogel droplets by mixing two immiscible liquid phases. Emulsification is the most widely used technique to fabricate microgels from a variety of materials such as alginate, agarose and collagen (Khademhosseini and Langer, 2007). Although the size can be controlled by adjusting viscosity of phases, the degree of agitation and inclusion of surfactants to modify the surface tension between two phases, spherical microparticles formed by conventional methods using shear or impact stresses are highly polydisperse in size. Monodisperse microparticles can be fabricated by microfluidic devices (Chu et al., 2007, Kim et al., 2007). Potential applications of nano- and microgels have been investigated as vehicles for drug delivery and delivery of encapsulated cells (Oh et al., 2008, Chung et al., 2012).

14.3 Scaffold-Free 3D Culture Systems

To overcome some of the limitations presented by scaffold-based tissue engineering, alternative approaches have been developed including scaffold-free formation of multicellular microtissues. All scaffold-free systems rely on cellular aggregation or self-assembly that are called spheroid since most of self-assembled aggregates are spherical in shape. In these methods, formation of aggregates from mono-disperse cells take place through a variety of cell adhesion molecules such as cadherins, tight junctions and gap junctions. The 3D culture systems have been used increasingly as they emulate *in vivo* tissue architecture and physiology more closely in comparison to monolayer culture systems. For instance, both parenchymal and non-parenchymal hepatocytes rapidly lose their normal phenotype and function following isolation and culture in monolayer, while the phenotype can be maintained when cells culture in 3D (Kim and Rajagopalan, 2010).

Various methods have been developed to form multicellular microtissues including spinner culture, hanging drop, pellet culture rotating wall vessels, liquid overlay, microfluidics and micro-moulded hydrogels. In pellet culture, centrifugation force is used to promote cell-cell adhesion and formation of aggregates. The main drawbacks include exposure of cells to shear forces and risk of low oxygen concentration and hypoxic cell death in core of large spheroids formed through this process (Jaehn et al., 2010, Giovannini et al., 2010, Markway et al., 2010, Li et al., 2011). Hanging drops is a gravity-assisted method in which a small volume of cell suspension is then inverted to allow formation of spheroids. Although spheroids size can be controlled in this method, the drawbacks include difficulties of extended culture and changing of the culture medium (Kelm et al., 2003, Timmins et al., 2005). To overcome the disadvantages, high-throughput 3D spheroid culture methods have been recently developed using 96- and 384-hanging drop arrays (Tung et al., 2011, Hsiao et al., 2012). To form spheroids, cell suspension can be also seeded onto a non-adherent surface such as alginate or agarose with gentle shaking to promote cell-cell interaction. Similar to other methods, liquid overlay methodologies rely on random interaction of cells, lack of control over size of spheroids is the main drawback (Carlsson and Yuhas, 1984, Landry et al., 1985). More recently, self-organizing capacity of cells have been utilized to form 3D structures resembling an organ known as organoids (Lancaster and Knoblich, 2014). Although formation of organized tissues have been reported previously in teratomas and embryoid bodies developed from pluripotent stem cells (Itskovitz-Eldor et al., 2000, Martin and Evans, 1975), formation of tubular and duct structures following embedding kidney and breast epithelia in laminin-rich ECM secreted by Engelbreth-Holm-Swarm tumour line, known as Matrigel, provide a new system in order to make microtissues resembling an organ *in vitro*. While organoids are powerful tools for modelling development and disease, they are highly heterogeneous and fail to exhibit structural organization of an organ due to lack of dorsal-ventral and anterior-posterior axes (Lancaster and Knoblich, 2014).

To form spheroids more uniformly *in vitro*, more advanced methodologies have been devised including the spinner flask, rotating wall vessel bioreactor, and microfluidics. The spinner flask is a method in which cell-cell aggregation is promoted through cell-cell collision in a cell suspension that is kept in constant motion using an impeller. The main disadvantages of this method are exposure of cells to shear forces and lack of control over size of spheroids (Nyberg et al., 2005, Han et al., 2006, Frith et al., 2010). The rotating wall vessels bioreactor is a technology that maintain the cells in microgravity environment by rotating a cell suspension along *x*-axis. To promote formation of spheroids, the rotation is initially slow and can be increased to higher speed as the spheroids growing in size. Lack of control over size of spheroids and low shear forces are the main drawbacks of this technology (Ingram et al., 1997, Hammond and Hammond, 2001, Carpenedo et al., 2007). In addition, microfluids can be exploited to form spheroids by channelling cell suspension to micro-chambers through a network of micro-channels. The process of cell aggregation by micro-rotational flow in microfluidic devices is highly controllable in terms of spheroid size and addition of soluble factors (Toh et al., 2007, Huang et al., 2009). Finally, the use of micro-moulded non-adhesive hydrogels is a relatively new method for formation of microtissues. In this technique, microtissues with more complex shapes than spheroids alone (Napolitano et al., 2007).

14.4 Modular Biofabrication

The modular biofabrication process is a 'bottom-up' approach and provides an alternative method of constructing large tissue by assembling smaller modules instead of conventional 'top-down' approach of seeding a large porous scaffold (McGuigan and Sefton, 2006). The modular approach aims to address the issues of low porosity and lack of interconnectivity in conventional tissue engineering. In the modular approach, the modules can be coated with endothelial precursors prior to assembly, which can form a network of interconnected channels following assembly of several modules (McGuigan and Sefton, 2006). Various methods have been developed to create modules including self-assembled aggregation (Dean et al., 2007), cell sheets (L'Heureux et al., 1998) and microfabrication of cell-laden hydrogels (Yeh et al., 2006). To build more complex tissue constructs, vasculature supporting cells such as smooth muscle cells (SMC) (Leung and Sefton, 2007), mesenchymal stromal stem cells (MSC) (Chamberlain et al., 2012) and therapeutic cells of interest, like islets (Gupta and Sefton, 2011) or cardiomyocytes (Leung and Sefton, 2010) can be embedded into modules. To build larger microtissues, these moulds can be assembled through various techniques such as stacking of layers (L'Heureux et al., 1998), random packing (McGuigan and Sefton, 2006) or directed assembly (Du et al., 2008). Both natural polymers such as collagen and synthetic polymers like PEG have been used for construction of microtissues through modular approach. Although PEG-based hydrogel systems offer more control over architecture of initial modules, exposure of cells to UV and toxic photoinitiator during crosslinking step can be considered as the major drawbacks.

14.5 3D Bioprinting

Over the past few decades, biomedical applications of additive manufacturing, known as 3D bioprinting, has been evolved as a new technology to overcome shortfalls of traditional tissue engineering procedures. Simultaneous layer by layer fabrication of living cells and biomaterials is the major advantage of 3D bioprinting over traditional approaches that can enable researchers to form tissues constructed with spatial control and precision positioning of living cells and biological materials.

14.5.1 Bioprinting Strategies

Microextrusion- (Cohen et al., 2006, Shor et al., 2009, Iwami et al., 2010), inkjet- (Roth et al., 2004, Xu et al., 2005, Cui et al., 2012, Xu et al., 2013) and laser-based (Barron et al., 2004b, Guillemot et al., 2010, Guillotin et al., 2010) technologies are the main technologies used for fabrication and patterning of cells and biological materials. The other features of 3D bioprinting including cell viability, surface resolution and biological components used for printing (Murphy and Atala, 2014).

14.5.1.1 Microextrusion Bioprinting Technology

Microextrusion printers function by deposition of a material on a substrate by a robotically controlled microextrusion head (Murphy and Atala, 2014). Normally, such a printer consists of various components including stage, a video camera for the *x*-*y*-*z* command, a fibre-optic light source for illumination of stage and activation of photoinitiator and a material handling and dispensing device. While the main advantages of microextrusion bioprinting technology lie in the ability to deposit a very high cell density, the cell viability is lower than inkjet-based bioprinting and varies from 40 to 86% based on extrusion pressure and nozzle gauge (Chang and Sun, 2008, Nair et al., 2009). Other challenging tasks that lie ahead of biomedical applications of microextrusion technology are increasing speed and resolution while maintaining cell viability and function (Murphy and Atala, 2014).

14.5.1.2 Inkjet Bioprinting Technology

Inkjet printers are the most common type of printer used for both biological and non-biological applications. Modification of commercially available 2D inkjet printers, including replacement of ink with a biological material and replacement of paper with an electronically controlled stage to provide control over the *z*-axis, led to the creation of first generation of inkjet printers for biological applications (Roth et al., 2004, Xu et al., 2005).

To create droplets, inkjet printers use thermal or acoustic forces (Murphy and Atala, 2014). In thermal inkjet printers, heating of the printer head is used to produce pulses of pressure to eject droplets from the nozzle. Although the temperature of printer head can reach up to 300 °C during the heating process, it has been shown that the overall temperature in the printer head rises by only 4–10 °C due to the short duration of heating (~2 μS) and has no substantial impact on the viability and functionality of printed mammalian cells (Xu et al., 2005, 2006) or stability of biological molecules (Goldmann and Gonzalez, 2000, Okamoto et al., 2000). While low cost and high printing speed are major advantages of thermal inkjet printers, low droplet directionality, non-uniform droplet size, frequent clogging of nozzle and risk of exposing biomaterials and cells to mechanical and thermal stress are major drawbacks (Murphy and Atala, 2014). Piezoelectric crystals that generate an acoustic wave have been used as an alternative approach in some inkjet printer heads to form liquid droplets at regular intervals (Tekin et al., 2008). In addition, acoustic radiation force generated by an ultrasonic field can be used to create liquid droplets for printing (Demirci and Montesano, 2007). Although the potential damage on cell membrane by 15–25 kHz frequencies used by piezoelectric inkjet bioprinters remains a major concern, the lack of exposure of cells to mechanical and thermal stress as well as generation of uniform droplet size and control over directional ejection are main advantages over an inkjet bioprinter using a thermal head.

The inkjet-based bioprinters offer various advantages including low cost, high printing speed and resolution and the potential to introduce concentration gradient of growth factors and cells throughout the 3D construct. However, the major drawbacks of technology including limitation of material viscosity, difficulty in achieving biologically relevant cell densities and the fact that biological materials need to be in a liquid format for droplet formation are the main obstacles for biological applications of inkjet-based bioprinters (Murphy and Atala, 2014).

14.5.1.3 Laser-Assisted Bioprinting Technology

Laser-assisted bioprinting is a modified version of a technique known as matrix assisted pulsed laser evaporation direct write (MAPLA DW), originally developed for deposition of inorganic material that was successfully adapted as a non-contact, nozzle free technology for rapid and accurate deposition of biological materials such as DNA, peptides and cells (Barron et al., 2004a, 2004b, Ringeisen et al., 2004, Colina et al., 2005, Hopp et al., 2005, Dinca et al., 2008). To print cell-containing materials, a focused laser pulses on the absorbing layer of the ribbon to generate a high-pressure bubble that transfers cell-containing materials from the ribbon and deposits on the collector substrate (Murphy and Atala, 2014). Despite various advantages such as ability to deposit high density of cells with microscale resolution and negligible effect on cell viability and function, the application of laser bioprinters have been limited due to high cost and complications and difficulties involved in preparation of individual ribbon, which is often required for each printed hydrogel or cell type (Murphy and Atala, 2014).

14.6 Tissue Modelling and Regenerative Medicine Applications of Pluripotent Stem Cells

Organ transplantation has been used successfully to replace and restore the function of vital organs such as liver, kidney and heart in medicine. Currently, organ transplantation is the sole treatment for end-stage disease, however, organ transplantation demands and immune matching of donor tissue often results in lengthy waiting times. Successful isolation and culture of embryonic and somatic stem cells and advances in the field of tissue engineering provide a potential alternative to restore the function of failed organs. Regenerative medicine is an interdisciplinary field of research that brings stem cell research and tissue engineering together to deal with the 'process of replacing, engineering or regenerating human cell, tissues or organ to restore or establish normal function' (Mason and Dunnill, 2008). It has been speculated that restoring the function of an organ can be achieved through various procedures including stimulating the patient's own repair mechanism, transplantation of pre-seeded scaffold with appropriate physical and mechanical properties with tissue-specific stem cells or even the possibility of growing tissues and organs for transplantation in the laboratory. More importantly, reprograming of somatic cells into pluripotent state by combination of four transcription factors (Takahashi and Yamanaka, 2006, Takahashi et al., 2007) has provided a unique opportunity to generate the cells from a patient's own body and may circumvent the requirement for immunosuppression.

Although 2D cell culture has been used to study stem cell proliferation and differentiation *in vitro*, the popularity of scaffold-free 3D culture systems has increased in recent years as the third dimension allows for higher deposition of ECM, greater cell-cell contact, and more efficient cell-matrix interactions compared to 2D systems. Embryoid bodies (EBs) are 3D aggregates of ESCs/iPSCs that can differentiate into all three germ layers (Itskovitz-Eldor et al., 2000). EBs have been produced by using various techniques such as hanging drops and by culturing on low adherence substrates, and have been used widely to study some aspects of embryonic development and mechanism of ESC/iPSC differentiation. EBs have been used to differentiate ESCs toward many lineages including neural (Zhang et al., 2001), haematopoietic (Dang et al., 2002) and cardiac cells (Kehat et al., 2001, Xu et al., 2002). Several strategies have been developed to generate uniform-sized EBs as it has been demonstrated that the size of EBs affects cell differentiation (Bratt-Leal et al., 2009). Although EBs can be formed with both morula-like structures and with a central cavity resembling the blastula stage (Abe et al., 1996), formation of organized and complex structures is limited due to difficulties in long-term maintenance of the EBs in culture. Moreover, heterogeneity of differentiated cells as a result of spontaneous differentiation following cell aggregation and formation of

EBs, limitation of nutrient and gaseous diffusion and difficulties in maintenance of cultured EBs are major disadvantages of this technique and have further limited their applications in stem cell and biomedical research.

More organized and complex tissue-like structures, organoids, have been formed *in vitro* by culturing stem cells in Matrigel ECM. Organoids are highly heterogeneous 3D structures that exhibit typical tissue architecture and can be used as a powerful tool for modelling disease and development. So far, several protocols have been established for *in vitro* generation of organoids for the brain (Eiraku et al., 2008), gut (Sato et al., 2009), retina (Eiraku et al., 2011), liver (Huch et al., 2013) and kidney (Takasato et al., 2014). Contrary to EBs, organoids can be propagated and maintained with relative ease in culture for long periods of time.

Although generation of organoids has been mainly established in mice, adaptation of methodology to form human organoids holds great potential. Organoids can serve as a valuable developmental model to explore biological principals that exhibit difference in human compare with animal models. For instance, intestinal organoids have been used to study the role of crypt niche in stem cell self-renewal and differentiation (Sato et al., 2011, Durand et al., 2012). Moreover, iPSC technology in conjunction with organoid methodology can be used as a valuable tool in disease modelling. Although disease modelling by the generation of organoids from patient-derived iPSCs is in its infancy, the field is rapidly evolving and has the potential to model other human genetic disorders such as retinitis pigmentosa, autism, epilepsy and schizophrenia (Lancaster and Knoblich, 2014).

14.6.1 The *In Vitro* Hepatic Models

Chronic liver disease and the subsequent loss of liver function is a major cause of morbidity and mortality. The number of liver failure cases has risen due to a number of factors including aging population of hepatitis patients, lack of vaccination against hepatitis C and emergence of new liver diseases such as steatohepatitis and non-alcoholic fatty liver disease (Bhatia et al., 2014). The number of people affected with viral hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV) is estimated to be 600 million worldwide (Gonzalez and Keeffe, 2011). In addition, alcoholic hepatitis develops in over one-third of heavy alcoholic drinkers. Chronic inflammation resulting from liver damage leads to formation of fibrotic tissue and consequently malignant transformation (Szkolnicka et al., 2013, Ignatius Irudayam et al., 2014). Although liver transplantation is considered the primary treatment for liver failure, the number of donor organs does not meet the demand of an increasing number of patients, despite improvements in organ allocation and pursuing new surgical routines such as split liver transplants and living-related partial donor procedures (Bhatia et al., 2014).

Cell-based therapies have held promise as an alternative approach, however, challenges stemming from complexity of liver structure and limited availability of human hepatocytes has hindered progress in this area. Contrary to many other fully differentiated cells, hepatocytes have remarkable capacity to replicate *in vivo* (Overturf et al., 1997). In fact, transplantation of hepatocytes has been investigated as a potential alternative to orthotopic organ transplantation through portal vein infusion or transplantation into other tissues including the mesentry, spleen, renal capsule and lymph node (Ponder et al., 1991, Gupta et al., 1999, Ohashi et al., 2005, Komori et al., 2012). Extensive research has focused on development of techniques to culture and maintain hepatocytes *in vitro*, due to limited availability of fresh hepatocytes. Like many other cell types, 2D culture configuration has been explored predominantly by culturing hepatocytes on ECM/polymer-coated dishes. To coat, various ECM proteins such as liver biomatrix, collagen type I, Laminin, and Matrigel have been explored (Reid et al., 1980, Bissell et al., 1987, Schuetz et al., 1988, Bissell et al., 1990, Moghe et al., 1997). It has been demonstrated that specific ECM proteins such as laminin, fibronectin and collagen type I and IV promote differentiation toward hepatic lineage and induce secretion of albumin (Flaim et al., 2005).

To further improve viability and functionality, other approaches have been explored, including culturing cells between two layers of ECM (Dunn et al., 1991), co-culture with other hepatic and non-hepatic cell types (Tukov et al., 2006) and co-culturing patterned hepatocytes on a microscale architecture surrounded by fibroblasts (Khetani and Bhatia, 2008).

Despite improvements in cell viability and functionality, especially in the case of ECM sandwich cultured cells, the shortcomings of the 2D platform encouraged exploration of 3D culture systems. Various techniques have been utilized to replicate hepatocytes and maintain their functionality *in vitro*, including culturing cells on coated surfaces, which encouraged spontaneous formation of spheroids, or methods using physical means to increase cell-cell contact such as hanging drop and culturing cells under rotational or rocking motions (Lin and Chang, 2008). Hepatocytes exhibit excellent viability and better maintain their functionality and phenotypes following formation of both homogenous and spheroids co-cultured with non-hepatic lineages over 2D cultured cells. It has been known that co-culture of various cells that normally exist in the same organ *in vivo* has beneficial effects on survival and maintenance of function in cells cultured in 2D *in vitro* culture systems (Clement et al., 1984). For instance, long term cultivation and functionality of parenchymal hepatocytes were reported following co-culture with non-parenchymal cells such as stellate cells (Gressner et al., 1995) and Kupffer cells (Hespeling et al., 1995). Despite the positive impact upon co-culture of cells in 2D, lack of appropriate architecture and spatial arrangement due to inherent limitation of 2D systems can be considered as limiting factors. As explained before, 3D culture systems provide a better environment for cells to reorganize themselves and re-arrange in a polarized manner. In addition, enhanced albumin secretion and cytochrome P450 activity have been observed in hepatocytes co-cultured with non-hepatic cell types under 3D culture condition compared to mono-cultured hepatocytes (Lu et al., 2005, Thomas et al., 2005, Kojima et al., 2011, Lee et al., 2013). However, spheroid culture of hepatocytes faces various challenges, including difficulty over controlling size, long lag period for formation of spheroids and adaptation for high-throughput screening (Bhatia et al., 2014). Advanced methodologies have been used to overcome these issues including using a spinner flask to reduce the lag phase (Glicklis et al., 2004), micro-imprinting (Fukuda et al., 2006), 2D microarrays (Otsuka et al., 2004) and microfluidics (Lee et al., 2013) to make uniform spheroids and making 3D culture amenable to high-throughput screening (Lin and Chang, 2008, Bhatia et al., 2014).

Despite progress in culturing primary hepatocytes, to circumvent the shortage of access to fresh primary human hepatocytes, extensive effort has been put into derivation of hepatocyte-like cells from pluripotent stem cells (HLCs) and iPSCs (iHLCs) (Hay et al., 2008a, 2008b, Basma et al., 2009, Si-Tayeb et al., 2010, Sullivan et al., 2010, Touboul et al., 2010, Szkolnicka et al., 2014, Zhu et al., 2014). Although safety concern over transplantation of HLCs and iHLCs, plus challenges ahead of clinical-scale production remain to be addressed, nevertheless, HLCs and iHLCs are considered as invaluable *in vitro* tools for drug discovery, toxicity testing, to model liver disease and to develop extracorporeal bioartificial liver devices and implantable therapeutic constructs for preclinical testing.

As the main metabolic organ in the body, liver is involved in the metabolism of fat, carbohydrates and proteins. Mutation in key proteins involved in metabolic pathway can lead to loss of function and consequently metabolic disorders. Pathobiology of these disorders can be studied at the cellular level following differentiation of iHLCs. So far, *in vitro* models of several metabolic disorders have been generated using this technology including hereditary tyrosinemia type 1, glycogen storage disease type 1a, progressive familial hereditary cholestasis multifactorial, Crigler–Najjar syndrome, α1-antitrypsin deficiency, familial hypercholesteromia and Wilson's disease (Ghodsizadeh et al., 2010, Rashid et al., 2010, Zhang et al., 2011). Reverse genetic engineering techniques can also be used to create heterozygous or homozygous mutant isogenic cell lines to bypass reliance on generation of patient-derived iPSCs to model disease *in vitro* and to study the function of various gene by creating homozygous knock-out mutant. Such an approach was taken to define function of human AKT2 (Hussain et al., 2011) and SORT1 genes (Ding et al., 2013). In addition, these *in vitro* systems can be used as a platform to test efficiency and safety genome editing tools such as zinc finger nucleases

(ZFNs), transcription activator-like effector nuclease (TALEN) and RNA-guided Cas9 nuclease from clustered regulatory interspaced short palindromic repeats (CRISPR) as potential therapeutic approaches.

Furthermore, HLCs and iHLCs are useful tools to evaluate the interaction between hepatocytes and liver-specific pathogens such as HBV and HCV specifically the innate immune aspect (Wu et al., 2012). HCV is a single stranded, positive-sense RNA virus which replicate in functionally mature hepatocytes. It has been shown that iHLCs expressed permissive factor miR-122 (Wu et al., 2012) and cell entry receptors occluding SRBI, CD81 and CLDN1 (Schwartz et al., 2012). It was demonstrated that HLCs support HCV full life cycle including the release of infectious virions (Roelandt et al., 2012, Zhou et al., 2014). Further investigation revealed that HCV triggered the type III interferon innate immune response in HLCs leading to a low level of infection and replication that can be rectified by modulating the innate immune response by inhibition of the JAK/STAT signalling pathway (Zhou et al., 2014).

HBV is a small DNA virus that selectively infects hepatocytes. The number of chronically HBV-infected patients is estimated to be around 400 million globally. Chronically infected patients are at risk of developing HBV-related complications such as cirrhosis and liver cancer. Several approaches have been pursued in quest for an *in vitro* model to study the pathogen-host interaction and to test anti-viral agents. It has been demonstrated that hepatoma cell lines such as Hep G2 and HepaRG are susceptible to HBV infection and support the HBV life cycle and release of virions (Chang et al., 1987, Sells et al., 1987, Gripon et al., 2002). However, hepatoma cell lines are not suitable models to study HBV life cycle and anti-viral agents due to disadvantages associated with these cell lines, including chromosomal abnormalities, heterogeneity in albumin expression and lower drug metabolism enzymes in comparison to primary hepatocytes. Despite development of *in vitro* models using primary hepatocytes (Shimizu et al., 1986, Gripon et al., 1988, Ochiya et al., 1989, Gripon et al., 1993), limited availability and phenotypic instability of primary hepatocytes after isolation and loss of phenotype following *in vitro* culture hinder wide utilization of these models. To overcome phenotypic instability, the co-culture of primary hepatocytes and iHLCs on micropatterned substrate has been investigated recently. The result indicated that a prolonged infection can be achieved using this approach. Similar to other observations, the infection was restricted by an innate immune response and could be enhanced by suppression of the immune response (Shlomai et al., 2014). More recently, isolation of fresh hepatocytes from chimeric humanized mice was proposed as an alternative approach to overcome limitation of access to fresh primary hepatocytes for an *in vitro* HBV infection model (Ishida et al., 2015). However, this approach is not cost effective and faces ethical concerns due to reliance on animal models to regenerate human primary hepatocytes.

Malaria is another widespread disease caused by a parasite from the *Plasmodium* family. Plasmodium has an obligatory liver stage that occurs early in human host infection. Liver-stage malaria is an attractive stage for eradication of disease from the host body, therefore, an *in vitro* liver-stage model for drug screening and finding new antimalarial drugs is imperative (Mazier et al., 2009). As described earlier, *in vitro* models using primary hepatocytes have advantages over models using hepatoma cell lines such as Hep G2 and HC 04 (March et al., 2013). Recently, an *in vitro* liver-stage malaria model was established by infection of iHLCs with *Plasmodium* sporozoites from rodent malaria species (*P. berghei* and *P. yoelli*) and human malaria species (*P. vivax* and *P. falciparum*) (Ng et al., 2015). In addition, mature iHLCs responded to antimalarial drug premaquine demonstrating usefulness of this model for antimalarial drug testing applications.

14.7 Applications in Drug Discovery and Toxicity

The development of new drugs is a laborious and costly process largely due to three main reasons. Primarily, the enormity of discovery of the lead molecule with unique qualities out of a large number of potential compounds. Secondly, cost of target validation, pharmacokinetic and pharmacodynamics analysis through a

series of preclinical *in vitro* and *in vivo* assays. Finally, the failure of drug candidates identified in the initial *in vitro* screening during clinical *in vivo* assessment. New approaches have been developed both for drug discovery and drug screening to provide more cost effective and biomimetic platforms, including *in vitro* cell culture assays. Here, the latest development will be discussed.

14.7.1 3D Culture Systems

Traditionally, cells grown in monolayers have been exploited predominantly as initial models to evaluate effectiveness and safety of new potential therapeutic compounds to bridge the gap between initial biochemical assays to identify lead compounds and preclinical animal tests. However, there has been a substantial shift towards using 3D models specially to model tumours in recent years, as they behave differently to 2D cultured cells and resemble the *in vivo* conditions more closely. The spheroids are heterogeneous, contain a proliferating cell population on surface and quiescent cells in the centre due to limited access to nutrient and oxygen (Mehta et al., 2012) and may contain necrotic areas in larger spheroids that resemble the *in vivo* structure of some tumours more closely (Yoshii et al., 2011).

Several studies show that more realistic outcomes can be achieved to study tumorigenic mechanism by 3D organization of cells for *in vitro* cancer study and how cells may respond to drug treatment (Breslin and O'Driscoll, 2013). A clear example of such a difference was elegantly exhibited following culture of SKBR-3 cells overexpressing HER2 oncogene in 2D and 3D (Pickl and Ries, 2009). Cells cultured in 2D formed a HER2 heterodimer with HER3, whereas cells grown as spheroids had HER2 homodimers and expressed a lower level of epithelial growth factor receptor (EGFR), activated Akt and showed enhanced phosphorylation of HER2, HER3 and EGFR. In addition, the response to a fixed concentration of trastuzumb, a monocolonal antibody that target HER2, was significantly different between cells cultured in 2D and 3D with a 16 and 48% reduction in proliferation, respectively, due to differential expression of surface markers and, as a result, intracellular signalling events (Pickl and Ries, 2009).

Hypoxia influences various aspects of tumour biology such as altered metabolism, resistance to cell death, angiogenesis, invasiveness, metastasis and genomic instability and is considered a negative prognostic factor (Wilson and Hay, 2011). Therefore, 3D models can be considered better to evaluate the effect of drugs intended for use on solid tumours since the core region of 3D models can experience hypoxic conditions. A clear example was presented by the difference in viability shown by 2D and 3D cultured A431. The H9 cell line was treated with the same concentration of tirapazamine (TPZ) and 5-fluorouracil (5-FU) (Tung et al., 2011). Cells cultured in 2D were more resistant to treatment with TPZ and showed 72% viability, while the viability was just 5% for cells treated with 5-FU. Conversely, cells cultured in 3D were more responsive to treatment with TPZ with 40% viability, whereas the viability of 3D cells treated with 5-FU were 75%. The difference was correlated with the attribute of TPZ as a hypoxic activated cytotixin, which works more effectively on cells cultured as 3D since the core of spheroids is hypoxic due to limitations in oxygen diffusion. Since the efficiency of chemotherapeutic agents is influenced by the rate at which the drug diffuses into a cancerous mass, the variation in response to 5-FU can be explained by limited availability of drug in 3D system compared to 2D cultured cells. Due to limitation in mass transfer, spheroids are closely mimic *in vivo* limited drug penetration. Despite prolong exposure, it has been shown that some drugs only penetrate to the depth of few cells at non-negligible quantities (Erlanson et al., 1992, Minchinton and Tannock, 2006).

Moreover, it has been shown that cells grown as spheroids exhibited polarized 3D architecture and were more resistant to apoptosis and treatment with chemotherapeutic agents (Weaver et al., 2002). Other factors for lower efficiency of anti-cancer drugs in 3D systems in comparison to 2D cultures include promotion of

cell survival following activation of signalling pathway like PI3K/Akt, Stat3 and NF-κB by intercellular cell contacts, impaired efficiency of anti-cancer drugs with selective toxicity on dividing cells due to reduction of cell proliferation rate in 3 D spheroids and binding of drug molecules to ECM and reduction in available molecules in ECM-rich 3D systems (Olive and Durand, 1994).

The effect of extracellular cues on cell behaviour is a well-documented phenomenon (Clark et al., 1991, Berrier and Yamada, 2007, Legate et al., 2009). There is evidence suggesting differential cellular response due to divergent signals that cells receive from microenvironment cues in 2D and 3D. An example is the study by Weaver and colleagues culturing non-malignant and malignant HMT-3522 breast cells in 2D and 3D using Matrigel (Weaver et al., 1997). While in 3D, non-malignant cells formed organized polarized acini similar to those found in a healthy breast, the β1-integrin overexpressing malignant cells formed loose and disorganized aggregates. Whereas the malignant cells phenotype was corrected following treatment with an antibody against the β1-integrin receptor, non-malignant cells went through apoptosis. More interestingly, similar results were not obtained on cells cultured in 2D emphasizing the importance of microenvironment cues on cell behaviour (Weaver et al., 1997). Although the 3D culture of cancerous cells has been used in various studies with promising outcomes to screen drugs, developing *in vitro* models using primary human cells is imperative for better accuracy in drug screening and preclinical testing.

14.7.2 Liver *In Vitro* Models for Drug Discovery, Toxicity, and Modelling Drug Metabolism

As the largest internal organ, the liver carries out a multitude of functions including glycogen storage, secretion of albumin, metabolism of bile acids and clearing xenobiotics and drugs from bloodstream to mention a few. Exposure to xenobiotics makes the liver susceptible to toxic injury (Szkolnicka et al., 2013). In fact, drug toxicity is one of the leading causes of liver injury and is estimated to be the second major cause of post-marketing drug withdrawal (Astashkina and Grainger, 2014). Since controversy around animal models as predictive models for man is rising, and by increasing pressure from regulatory agencies over reduction or obviation of animal models from drug development process from an ethical viewpoint, the need for a highly predictive *in vitro* toxicity model is of outmost importance in current preclinical evaluations (Mann, 2015).

Currently, *in vitro* models using human and animal primary hepatocytes are viewed as the most physiologically relevant models, however, phenotypic instability and scarcity are the major disadvantages, as described earlier. Although immortalized and hepatoma cell lines have been examined as potential alternatives for *in vitro* evaluation of drug screening and toxicity, their poor functionality, karyotypic instability and their higher tolerance to toxicological insults compare with primary hepatocytes limits large-scale application (Allain et al., 2002, Guillouzo et al., 2007). Despite remaining caveats, HLCs generated from PSCs and iPSCs offer reat promise as an alternative to developing *in vitro* cell-based for drug screening and toxicological preclinical testing.

In addition, it has been suggested that genetic factors may result in different responses due to difference in drug absorption, distribution, efficacy and exertion among patients, which is interesting in context of race and ethnicity (Xie et al., 2001). It has been estimated that about 15–30% of inter-individual differences observed in drug metabolism and side effects resulted from genetic variation within xenobiotic-metabolizing enzymes and transporters encoding genes (Eichelbaum et al., 2006). In addition, mutations and polymorphisms that affect genes involved metabolic activity can have profound effect on liver function and increase the risk of liver injury. In most populations, enzymes involved in phase I (members of cytochrome P450 family) and phase II drug metabolism are polymodels, resulting in variation in responses to drug (Szkolnicka et al., 2013). Therefore, developing liver models can help to gain a better understanding of polymorphisms and the role of genetic background on drug metabolism and liver toxicity.

14.7.3 Microfluidics

Discovery of drugs requires reliable and fast methods to find and test probable candidates. In order to reduce the cost and increase the number of hits, the field of drug discovery relies on automation and high throughput screening. The miniaturization of process by microfluidic chips can be considered a major breakthrough, which can increase discovery speed and provide automation opportunities. Reduction in sample consumption, possibility to integrate miniaturized functional units including pumps, valves, sensors and detectors and flexibility for adaptation with highly sensitive and common detection techniques such as mass spectroscopy, electrochemical methods and optical methods including chemoluminescence and fluorescence, make microfluidic chips an ideal drug discovery tool.

Microfluidics technology can be used in drug discovery in various ways. Reproducible generation of homogenous femtolitre to nanolitre droplets vesicle can be used in screening experiments such as crystallization and molecular evaluation (Kuhn et al., 2002, Tawfik and Griffiths, 1998). Plug-based microfluidic systems can be utilized in structure-based drug discovery as lower volumes result in a reduction in the amount of the required starting materials. For instance, 10 nl aqueous droplets were generated by a microfluidic chip to determine the optimum crystallization condition (Zheng et al., 2003) and evaluation of crystal quality by X-ray diffraction (Zheng et al., 2004). Aqueous droplets can also be used to form cell-size compartment to engulf genes, RNAs and encoded proteins for molecular evaluation of candidate drugs (Tawfik and Griffiths, 1998, Dittrich et al., 2005).

Furthermore, microfluidics chips can be adapted to make biomimetic organ-on-chip platforms, which recapitulate physiological microenvironment for drug screening and testing. To date, lung, heart, liver, gut, kidney, breast and blood vessels biomimetic organ-on-chips have been designed with potential application in drug discovery and toxicity testing (Bhise et al., 2014). Advances in microengineering have opened new horizons to create more complex and advanced *in vitro* models resembling 3D organ-level structures. For instance, a liver-on-chip device was created to co-culture liver parenchymal cell with fibroblasts to maintain liver function (Kane et al., 2006), or to provide interstitial tissue flow by stacking a two-patterned silicon mould and soft lithography film to integrate a micropatterened vasculature network with liver parenchymal cells (Carraro et al., 2008).

14.8 Conclusions

Despite decades of research, the hepatocyte microenvironment and mechanism of cell replication are not fully understood. As a result, hepatocytes rapidly lose their ability to proliferate in cell culture and demonstrate a compromised phenotype. Therefore, further research is required to gain a better understanding of the hepatocyte interactions with their *in vivo* microenvironments, including their interactions with the non-parenchymal cell compartment and extra-cellular matrices. Recent advances in development of *in vitro* hepatocyte models using pluripotent stem cells provide a platform to improve current models for *in vitro* drug screening and preclinical evaluation. In addition, *in vitro* generation of liver tissue in combination with advanced technologies, such as bioprinting, provides future hope as a cell therapy for treating end stage liver failure.

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15

Characterization and Therapeutic Uses of Adult Mesenchymal Stem Cells

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15.1 Introduction

In the past two decades, adult mesenchymal stromal cells (MSCs), also referred to as mesenchymal stem cells (MSCs), have received great attention in attempts to use them for various applications, including tissue modification, repair, and regeneration on cell-based therapies (Kolf et al., 2007; Hu et al., 2008). These cells can be isolated from bone marrow and fat cells. Characteristics of MSCs include fibroblast-like morphology, high proliferation rate, attachment to cell culture dishes, colony formation, and capacities to differentiate into different mesenchymal lineages. They have been applied clinically to control autoimmune and graft-versus-host diseases (Chamberlain et al., 2007; Le Blanc et al., 2008; Tian et al., 2008; Djouad et al., 2009), myocardial infarction (Shen et al., 2015; Strauer et al., 2001), cartilage defects and osteoarthritis (Wang et al., 2015) and others. In pre-clinical studies, MSCs have been shown to provide protection against radiation-induced liver injury (Francois et al., 2013), promote healing in an irradiated murine skin wound (Hao et al., 2009; Kiang and Gorbunov, 2014), improve survival in irradiated mice (Hu et al., 2010; Kiang and Gorbunov, 2014; Kiang, 2016), mitigate the gastrointestinal syndrome in mice (Saha et al., 2011), restore the intestinal mucosal barrier in irradiated mice (Garg et al., 2014), reduce cartilage defects and osteoarthritis (Wang et al., 2015), lung injury (He et al., 2015), polycystic kidney disease (Franchi et al., 2015), acute graft-versus-host disease (Yang et al., 2015), myocardial infarction (Ghostine et al., 2002; Fukushima et al., 2008), stroke (Tsai et al., 2011), Huntington's disease (Linnares et al., 2016), and cognitive dysfunction (Acharya et al., 2015).

It is evident that MSC modification can reinforce MSC capabilities. Abdel-Mageed et al. (2009) reported that superoxide dismutase (SOD) gene-transfected MSCs improved survival in irradiated mice.

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However, other reports showed MSCs alone did not improve survival in irradiated mice (Abdel-Mageed et al., 2009; Kiang and Gorbunov, 2014; Kiang, 2016). MSCs overexpressing the angiotensin-converting enzyme 2 (ACE 2) result in a further improvement after lung injury (He et al., 2015).

Recent understanding on the cellular and molecular signaling activations on adult MSCs has provided new insights into their potential clinical applications, particularly for tissue repair and regeneration. This chapter focuses on these advances on MSC characterization and therapeutic uses.

15.2 MSC Characterization

MSCs are present in nearly all postnatal tissues or organs, including umbilical cord blood (Erices et al., 2000; Lee et al., 2004), the placenta (In't Anker et al., 2004; Yen et al., 2005; Kern et al., 2006), fat tissue (Gimble et al., 2007), and bone marrow (Tropel et al., 2004; Kiang et al., 2016), among others. MSCs represent a progenitor population with multiple differentiation potentials (Jackson et al., 2001; Kocher et al., 2001; Dayoub et al., 2003; Jorgensen et al., 2003; Kraitchman et al., 2003; Tocci and Forte, 2003; Tuan et al., 2003; Dominici et al., 2006; Shipounova et al., 2012). With the advantage of autologous transplantation that avoids the immune rejection and ethical concerns, MSCs have great application prospects in personalized treatment of diseases (Lodie et al., 200; Geng, 20032; Gronthos et al., 2003). Purification of MSCs is important for their efficacy. MSCs have markers expressed on cell membrane surface. Therefore, these markers are served to verify the identity of MSCs, while cell surface markers present in other type of cells such as hematopoietic progenitor stem cells are used as negative marker controls.

15.2.1 MSC Negative Markers

MSCs have been identified as colony-forming unit-fibroblasts (CFU-Fs) by Friedenstein and colleagues in 1970, and Pittenger and colleagues described in detail the tri-lineage potential of MSCs in 1999. Since then, our understanding of these cells has advanced. MSCs are multi-potent, adherent, and can be isolated from many adult tissue types. To ensure the isolated cells are MSCs, there is a consensus (Kolf et al., 2007) that MSCs do not express glycophorin-A (an erythroid linerage marker), CD11b (an immune cell marker), CD31 (an endothelial and hematopoietic cell maker), CD34 (a primitive hematopoietic stem cell marker), CD45 (a marker of all hematopoietic cells), and CD117 (a hematopoietic stem/progenitor cell marker).

CD11b, CD31, CD45, and CD117 are certain MSC negative markers in both human MSCs and murine MSCs, while CD34 surface marker is certainly negative in human MSCs. In contrast, CD34 has also been found to be positive in murine MSCs (Kolf et al., 2007). Because of this uncertainty of markers in murine MSCs, multiple negative and positive surface markers are used for murine MSCs verification.

15.2.2 MSC Positive Markers

MSC positive markers are available to ensure that isolated cells are MSCs. There are Stro-1, CD10, CD13, CD29, CD44, CD73, CD90/Thy-1, CD105, CD106 (i.e., VCAM-1), CD271/NGFR, Flk-1/CD309, and Sca-1, Stro-1 is the best-known MSC marker by far, because Stro-1 negative cells do not form colonies (Simmons et al. 1991). However, its expression in MSCs is gradually lost during culture expansion (Gronthos et al., 2003), with yet unidentified mechanism(s). It is unclear whether the loss of stro-1 marker will result in the loss of colony capability. Nonetheless, MSCs are always identified with Stro-1 in conjunction with other MSC positive and negative marker proteins.

Abdel-Mageed et al. (2009) identified MSCs with positive expression of CD13, CD29, CD44, CD105, and Sca-1 and negative expression of Thy-1.2, c-kit, CD11b, CD19, CD31, CD34, CD45, CD73, and CD135,

while Francois et al. (2013) identified MSCs with positive expression of CD105 and CD73 and negative expression of CD45. Saha et al. (2011) identified MSCs with positive expression of CD29 and CD105 and negative expression of CD11b and CD133. Felka et al. (2014) identified MSCs with positive marker proteins CD73, CD90, CD105, and CD146, and negative marker proteins CD11b, CD14, CD34, and CD45. Franchi et al. (2015) characterized MSCs with positive CD90 and CD29 expressions and negative CD45 and CD11b/c expression. Yang et al. (2015) characterized MSCs with positive Sca-1, CD29, CD44, CD90.2, and Flk-1 expression and negative CD117 and CD34 expression. In our laboratory, MSCs are identified with positive expression of Strol-1, Sca-1, CD44, and CD105 and negative expression of CD3 and CD34. MSCs are further confirmed with colony formation (Kiang and Gorbunov, 2014).

15.2.3 MSC Self-Renewal and Maintenance

MSCs are capable of self-renewal without differentiation. These cells express the embryonic stem cell gene markers oct-4, sox-2, and rex-1 (Izadpanah et al., 2006) that are involved in repressing differentiation genes (Boyer et al., 2006). In addition, the presence of leukemia inhibitory factor (LIF: Jiang et al., 2002; Metcalf, 2003), fibroblast growth factors (FGF, Tsutsumi et al., 2001; Zaragosi et al., 2006), and mammalian homologues of *Drosophila* wingless (Wnt; Boland et al. 2004; Kléber and Sommer, 2004) is observed. Hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and/or cytokines support MSC stemness in an MSC niche (Kolf et al., 2007). Beta-catenin, an extracellular matrix protein for anchoring cells in place, is thought to be involved in Wnt regulation of MSC self-renewal (Bienz, 2002).

The underlying molecular mechanisms of MSC differentiation still remain unclear, but some of factors for tissue repair and regeneration have been unfolded. MSC differentiation needs to be induced clinically by administration of transforming growth factor-beta (TGF-beta), bone morphogenetic protein (BMF), growth and differentiation factor (GDF: Chen et al., 2004), and Wnt ligands (Hartmann, 2006) for chondrogenesis, tenogenesis, and osteogenesis, peroxisome proliferator-activated receptor gamma (PPARgamma) for adipogenesis (Nuttal and Gimble, 2004), and Notch 1 for myogenesis (Dezawa et al., 2005), respectively. To keep in mind, the differentiation signal must find its way to the MSC niche for initiation of differentiation. The detailed process requires further studies (Kolf et al., 2007).

15.2.4 MSCs Proliferate in Hypoxia Faster than in Normoxia

The proliferation rate of MSCs depends on the environment where these cells reside. Cells proliferated at an approximate rate of 0.4458×10^5 cells/h and 1.2067×10^5 cells/h under normoxia and hypoxia, respectively, when approximately 1×10^6 cells were seeded and cultured (Kiang, Ho, Smith, unpublished data). It was reported that it took 32–35 h (De Luca et al., 2013; Kiang and Gorbunov, 2014) to double MSC numbers under normoxia. The results suggest that MSCs proliferate better in low oxygen tension, a phenomenon similar to cancer stem cells (Jiang et al., 2015), yet the underlying mechanisms leading to this accelerated cell proliferation is not identified. Figure 15.1 shows the difference in size of the colonies under each normoxia and hypoxia (Kiang et al., unpublished data).

15.2.5 MSCs Kill Bacteria by Autophagy

MSCs normally have relatively high amounts of constitutively expressed HSP70 and NF-keppaB-p65, and a detectable amount of NAD^+-dependent deacetylase sirtuin-3 (Sirt3). Significant increases in heat shock protein 70 kDa (HSP70), NF-keppaB-p65, Sirt3, and matrix metalloproteinase-3 (MMP3) were found, when MSCs were exposed to 12 Gy but not 8 Gy. Sirt3 is a mitochondrial stress-response protein. Increases in Sirt3

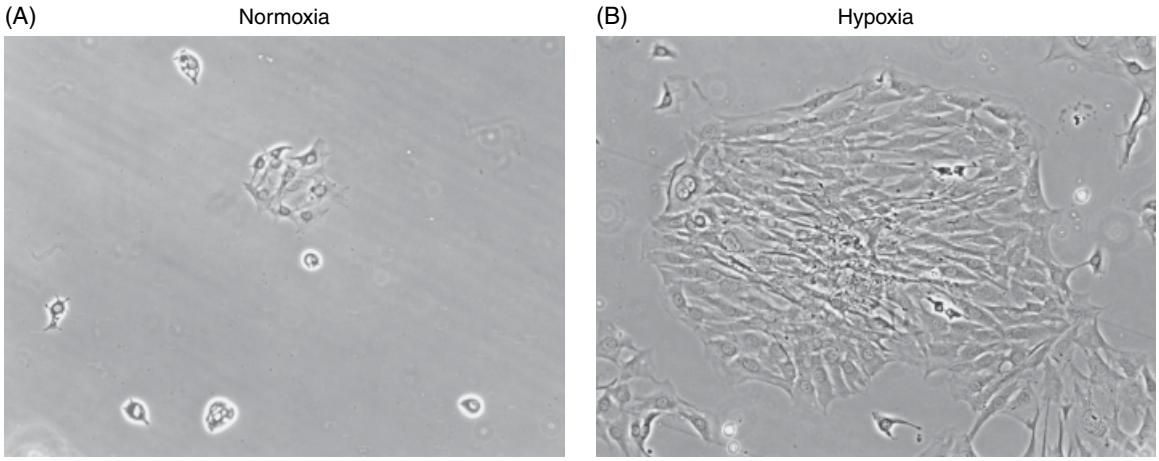

Figure 15.1 Hypoxia promotes mesenchymal stem cell colony formation. *MSCs were seeded in 4-well chambers at 4500 cells per well (Lab-Tek Chambered #1.0 Borosilicate Coverglass System, Fisher, Rochester, NY). On day 10, the 4-well chambers were rinsed with phosphate-buffered saline (PBS) prior to the addition of methanol. After sitting for 5 minutes, the chambers were again rinsed with PBS. The well cover was removed, and when dry, 1 drop of mounting medium (Vector Laboratories, Inc.; Burlingame, CA) was then added to each well and a cover slip was applied. For analysis, a fluorescence microscope (Zeiss; Ontario, Canada) and Axiovision software were used to image the MSCs. (A) Representative colony under normoxia. (B) Representative colony under hypoxia*

expression suggest that radiation induces stress to mitochondria. Caspase-3, a marker for caspase-dependent apoptosis, was not detected in irradiated MSCs, suggesting that no apoptosis takes place in MSCs after irradiation. Radiation induced significant increases in light chain 3 (LC3) expression, a marker of autophagy, detected by Western blotting and LC3-containing autophagy vacuoles displayed by immunofluorescent staining, suggesting the presence of upregulation of autophagy defense machinery.

It has been demonstrated that radiation induces systemic bacterial infection (Kiang et al. 2010; Fukumoto et al. 2013). In bone marrow, the immune homeostasis and defense responses to blood pathogens are mediated by the marrow-blood barrier, which consists of endothelial, reticuloendothelial, and mesenchymal stromal cell lineages (Owen and Friedenstein, 1988; Krebsbach et al., 1999; Balduino et al., 2005; Greenberger and Epperly, 2009). When MSCs were exposed to radiation or in combination with a Gram-negative *E. coli* challenge (5×10^7 bacteria/ml), increases in lysosomal-associated membrane protein 1 (Lamp1), small ubiquitin-related modifier 1 (SUMO1), collagen III, MMP3, MMP13, and p62/SQSM1 were observed 24 h after irradiation or combined with *E. coli* challenge. MSCs performed extensive phagocytosis and inactivated bacteria in autolysosomes (Gorbunov et al., 2013a). When MSCs were challenged with Gram-positive *S. epidermidis* (5×10^7 bacteria/ml) for 3 h, the cells displayed remarkable resistance to the bacterial challenge and sustained confluence over the period of observation. Similar observations to that with *E. coli* challenge (Gorbunov et al. 2015) or lipopolysaccharide (LPS) exposure (Gorbunov et al., 2013b) were found as well. We postulate that these pro-survival pathways activated in MSCs *in vitro* could be a part of adaptive responses employed by stromal cells under septic conditions. These results also suggest that MSCs can contribute to the innate defense response to radiation injury.

15.2.6 MSCs Exhibit Mitochondrial Remodeling

Radiation results in bacterial infection (Kiang et al., 2010). Radiation or bacterial challenge of MSCs results in alteration of the mitochondrial network. Transmission electron microscopy and immunofluorescence microscopy showed that the normal mitochondrial network is a combination of round and elongated organelles containing discrete cristae at high densities. Mitochondrial fusion and fission take place when necessary.

Using transmission electron microscopy, the bacterial challenge resulted in extensive mitochondrial swelling and cristae fragmentation 5 h post-challenge. The entire mitochondrial body almost became reticular by 24 h post-challenge. This structure re-arrangement and fragmentation were triggered by increased expression of immunity-related GTPase family M (IRGM) and inducible nitric oxide synthase (iNOS). Bacterial challenge induced dynamin-related protein 1 (Drp1, a marker of mitochondrial fission) translocation from cytosol to mitochondria, leading to activation of PTEN induced putative kinase 1- parkin RBR E3 ubiquitin protein ligase (PINK1-PARK2) to initiate mitophagy to degrade fragmented mitochondria (Gorbunov et al., 2015). Using Western blotting, proteins of mitofusin-1 (Mfn1, a marker of mitochondrial fusion), PINK1, and PARK2 in MSCs were significantly elevated 24 h after the bacterial challenge. The HSP70 basal level was not affected and no caspase-3 was detected in these cells (Gorbunov et al., 2015). These results suggest that mitophagy but not caspase-dependent apoptosis in MSCs occurs after the bacterial challenge. It is warranted that caspase-independent apoptosis caused by molecular pathways involving with apoptosis-inducible factor (AIF) or by senescence signals of protein 16 (p16) and beta-galactosidase (beta-gal) in MSCs after bacterial challenge shall be explored. MSCs exposed to ionizing radiation alone also show mitochondrial fission and subsequent fusion as well as mitophagy (Gorbunov et al., 2015).

15.2.7 MSCs and Signal Transduction

In vivo, radiation activates AKT, JNK, and p38 proteins (Kunwar et al., 2012). Felka et al. (2014) reported that NO activated c-Raf, JNK, p38, p53, and a nuclear factor E2-related factor (NRF2)-associated stress response, which may have detrimental consequences for bone remodeling or bone regeneration. *In vitro*, as shown in Fig. 15.2, our laboratory (Kiang et al., unpublished data) found that γ-radiation at 6 Gy decreased AKT activation (Fig. 15.2A) and increased JNK activation (Fig. 15.2B) in MSCs. Meanwhile, radiation reduced the cellular ATP (Fig. 15.3A) and mitochondrial ATP (Fig. 15.3B). Therefore, one can postulate that priming MSCs to elevate pro-survival signaling molecules may enhance MSCs' multi-functionality as a therapy.

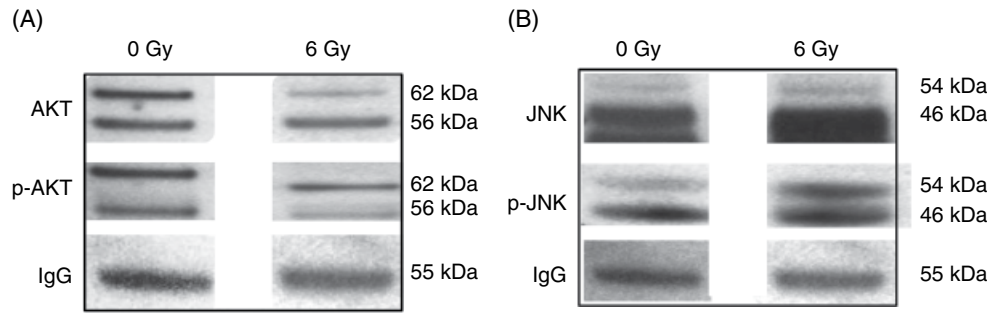

Figure 15.2 Radiation decreases AKT activation and increases JNK activation in mesenchymal stem cells (MSCs). *MSCs were irradiated at 4 or 6 Gy at 0.6 Gy/min with ^{60}Co-γ photons. Cell lysates were collected on day 2 after irradiation. AKT and JNK and their phosphorylation were detected using immunoblotting analysis. (A) Representative gels of AKT and its phosphorylation. (B) Representative gels of JNK and its phosphorylation*

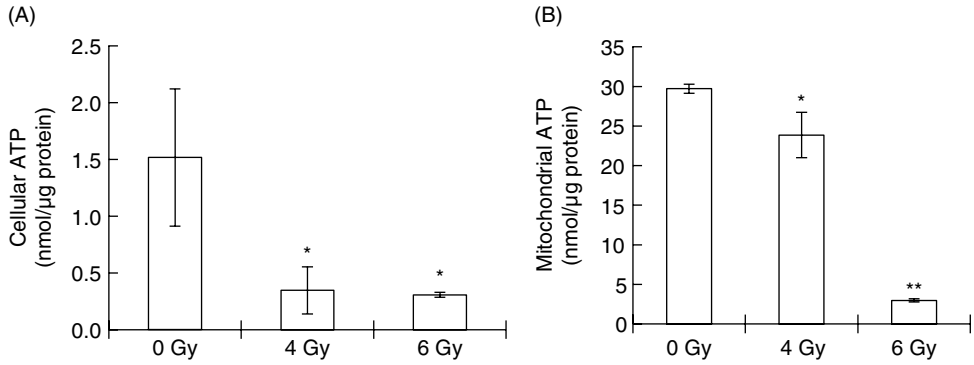

Figure 15.3 Radiation reduces ATP in cellular and mitochondria of mesenchymal stem cells (MSCs). *MSCs were irradiated at 4 or 6 Gy at 0.6 Gy/min with ^{60}Co-γ photons. Cells were collected on day 14 after irradiation. To fractionate intact mitochondria, a reagent-based method was employed using the Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific, Inc.; Waltham, MA). Cultured cell pellets were gently lysed using a proprietary formulation resulting in maximum yield of mitochondria with minimal damage to integrity. After collecting mitochondrial and cellular fractions, mitochondrial and cellular ATP levels were measured using the ATP Bioluminescence Assay Kit HS II (Roche; Manheim, Germany). Luminescence was measured with a TD-20/20 luminometer (Turner Designs; Sunnyvale, CA). Data were normalized to total protein. ATP in cell (A) and mitochondria (B) was measured. *p < 0.05 vs. control; **p < 0.05 vs. 4 Gy, determined by Student's t-test*

15.3 MSCs and Tissue or Organ Therapy

Organ dysfunction and failure have been serious problems for patients with various kinds of diseases, aging degeneration/dysfunction, accidental trauma, or hereditary disorders. Organ transplantation is one of choices for repair or clinically symptomatic amelioration. MSCs have been targeted to repair the problem using the laboratory animal models to develop methodologies and strategies. There are promising reports available.

15.3.1 MSCs Improve Acute Lung Injury

Acute lung injury is to have the diffuse damage of lung vascular endothelial cells and alveolar epithelial cells and an excessive inflammatory response in the lung (Ware and Matthay, 2000). It is evident that the renin-angiotensin system plays an essential role in causing this syndrome, because of an increasing level of angiotensin II generated by the angiotensin-converting enzyme (ACE). He et al. (2015) reported that ACE2 gene transfected MSCs (5×10^5 cells/mouse) were injected into male wild-type and ACE2 knockout C57BL/6 mice that had been rendered to have acute lung injury. ACE2 gene-transfected MSCs transplantation 24 h and 72 h later resulted in alleviation of the lung histopathology and presence of anti-inflammatory effects. The transplantation also reduced pulmonary vascular permeability, improved endothelial barrier integrity, and normalized lung eNOS expression. The report further indicates that ACE2 gene-transfected MSCs were recruited into the injured lung and enhanced local expression of ACE2 protein, thereby leading to decreases in angiotensin II levels accumulated in the lung.

The results in an injured lung from He et al. (2015) included decreases in neutrophil counts, IL-1beta and IL-6, increases in IL-10, and decreases in pulmonary edema. These results are consistent with previous studies in that the MSCs were mainly taken up by the injured lung 4–7 days post-injection (Anjos-Afonso et al., 2004). This improvement in acute lung injury in mice makes MSCs a potential therapy for treating this devastating clinical syndrome.

15.3.2 MSCs Improve Renovascular Function in the Kidney

Hereditary polycystic kidney disease is one of the most common lethal monogenic genetic diseases of man (Torres and Harris, 2007; 2009). There is no accepted treatment for this disease. Using a rodent experimental model, Franchi et al. (2015) intrarenally infused MSCs at 2.5×10^5 cells in 250 µl of PBS into female Sprague–Dawley rats that expressed polycystic kidney disease. Four weeks later, MSCs preserved vascular density and glomeruli diameter, reduced fibrosis, and preserved the expression of proangiogenic molecules. The benefit was observed up to 4 weeks after a single MSC infusion, suggesting that the cell-based therapy constitutes a novel approach in polycystic kidney disease, a lethal disease.

15.3.3 MSCs Effectively Treat Articular Cartilage Defects and Osteoarthritis

Sports injuries, accidental trauma, aging, osteoarthritis, necrosis of subchondral bone, or arthritis can cause articular cartilage damage. There is not only very limited for repair but this may also trigger progressive damage and joint degeneration. Current treatments for this kind of damage are primarily to alleviate symptoms, reduce pain, and control inflammation. But the treatments do not control the progressive degeneration of joint tissues (Schroeppel et al., 2011). Tissue engineering-based cartilage repair is a major research and was first reported by Brittberg et al. (1994), but the result was associated with undesirable disadvantages such as presence of fibrocartilage instead of hyaline cartilage. Therefore, MSCs are targeted. MSCs have an immunomodulatory property, can attenuate tissue injury, inhibit fibrotic remodeling and apoptosis, promote angiogenesis, stimulate stem cell recruitment and proliferation, and reduce oxidative stress (Wang et al., 2015). The mediators important during tissue remodeling are matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). MSCs secret TIMPs that protect implanted cells via TIMP-mediated inhibition of MMP activity (Lozito and Tuan, 2011).

The chondrogenic potential of MSCs was first reported by Ashton et al. (1980). MSCs were delivered to a defective site by direct intra-articular injection or via a scaffold. There were MSC studies in animals (Wang et al., 2015). Currently, 20 clinical trials are out there (see www.clinicaltrials.gov). To date, the literature contains information on 15 clinical trials or case reports with follow-up durations of at least 6 months, including pain measurements, assessment of range of motion, and MRI after patients receiving single injections of MSCs at doses of 50×10^6 cells or 150×10^6 cells. Allogenic and autologous MSCs are similarly effective (Tay et al., 2012). There is one clinical study reporting that single MSC injections did not cause any problem up to 2 years later and no abnormal tissue growth was apparent (Vangsness et al., 2014).

15.3.4 Differentiated MSCs Improve Myocardial Performance

Cardiovascular disease, in particular coronary heart disease, is the main disease type causing the majority of deaths. The damage to heart wall is irreversible. The current pharmacological treatments, surgical measures, and heart transplantation are in practice with known disadvantages. Orlic et al (2001) reported that C57BL/6 male mice were injected s.c. with recombinant rat SCF, 200 µg/kg/day, and recombinant human G-CSF, 50 µg/kg/day once a day for 5 days before and 3 days after ligation of the coronary artery. Bone marrow cells (BMC) were mobilized to form 15×10^6 new myocytes, decreased mortality by 68%, and reduced infarct size by 40%.

The differentiation of myocardial cells from MSCs can be induced by biochemical, myocardial microenvironment, genetic modification, and traditional Chinese herbs (Shen et al., 2015), which provided a promising perspective to cell treatment on cardiac diseases. Strauer et al. (2001) reported the first case of the autologous MSCs transplantation for acute myocardial infarction in clinical trials. This team transplanted 1×10^7 autologous MSCs into an infracted artery by catheter. The transplantation was safe and preliminarily effective. Ghostine et al. (2002) injected 5×10^4 skeletal myoblasts by intramyocardial delivery system into

sheep and months later the cardiac function was improved. Fukushima et al. (2008) injected 5×10^6 GFP-expressing skeletal myoblasts from male Sprague–Dawley rats into female Sprague–Dawley rats with myocardial infarction by either retrograde intracoronary or intramyocardial routes. These skeletal myoblasts improved cardiac performance and physical activity, associated with reduced cardiomyocyte-hypertrophy and fibrosis, further supporting the effectiveness of differentiated stem cells. However, to satisfy the clinical usage, it needs to ameliorate the conditions of induction and to further improve the differentiation efficiency. More investigation with a larger sample size is still necessary.

15.3.5 MSCs Improve Radiation-Induced Damage in the Intestinal Mucosal Barrier

Radiation is known to induce intestinal damage. Garg et al. (2014) reported that male CD2F1 mice were subjected to a dose of 8 Gy total body irradiation (Cs-137 irradiator, 1.35 Gy/min). Within 4–6 h after irradiation these mice received an intravenous injection of 2×10^7 bone marrow cells (BMCs) supplemented with 1×10^7 spleen cells. The transplantation accelerated peripheral blood counts, enhanced the recovery of intestinal immune cell populations in jejunum mucosa, reduced intestinal permeability, reduced IL-1α increases, restored IL-6, IL-10, and IL-12 concentrations, and modulated the expression of Claudin-2 and -4 (tight junction proteins). Since whole BMCs were injected, whether MSCs were responsible for mitigation of intestinal mucosal barrier damage remains unclear and needs to be further studied. Whole bone marrow transplantation also showed the significant survival improvement in female B6D2F1 mice after irradiation (Ledney and Elliott, 2010).

Saha et al. (2011) reported that male C57BL/6 mice were irradiated at 10 Gy (total body irradiation) or 16–20 Gy (abdominal irradiation) with a 320 KvP, Phillips MGC-40 orthovoltage irradiator (0.72 Gy/min). These mice were then intravenously injected with 2×10^6 MSCs per mouse at 24 and 72 h after irradiation. All MSC administered mice survived from 10 Gy or 16–20 Gy for more than 25 days whereas irradiated mice administered with either the enriched myeloid fraction or the non-myeloid fractions failed to improve survival. MSCs induced crypt interstitial stem cell (ISC) regeneration, restitution of the ISC niche, and xylose absorption. R-Spondin1, KGF, PDGF, FGF2, and anti-inflammatory cytokines (G-CSF and Gm-CSF) were elevated in serum, while inflammatory cytokines (IL-6, IL-10, IL-12, and IL-17) declined.

15.3.6 MSCs Repair Radiation-Induced Liver Injury

Radiation induces liver injury that can be detected by elevation of AST and ALT. Francois et al. (2013) reported that NOD/SCID mice were irradiated with Cs-137 at 3.2 Gy (1.85 Gy/min) and then intravenously administered with 5×10^6 human MSCs in 0.1 mL 1X PBS. They indicated that MSC administration alone did not produce liver toxicity. MSC transplantation restored plasma urea, reduced plasma AST and ALT, and decreased the oxidative stress indicated by malondialdehyde formation.

They reported that stromal cell-derived factor 1 (SDF1) secreted by cells within injured tissues and its receptor C-X-C chemokine receptor type 4 (CXCR4) were necessary for the MSC migrating to damaged tissues. Livers of MSC administered mice displayed high levels of SDF1 and CXCR4 with reduction of mir-27b after irradiation. The latter is known to downregulate SDF1. It took 15 days for MSCs to differentiate into the hepatocyte phenotype as indicated by measuring liver specific genes such as CK18, CK19, and AFP (Francois et al., 2013).

15.3.7 MSCs Accelerate Radiation-Induced Delay in Wound Healing

It is evident that radiation delays skin wound healing (Hao et al., 2009, Kiang et al., 2012). Hao et al. (2009) report that male Sprague–Dawley rats were exposed to 6 Gy of ^{60}Co gamma-ray (0.31 Gy/min) followed by a full-thickness excisional skin-wound (2% total body surface area). Then 1×10^7 recombinant adenovirus

Adv-hPDGF-A/hBD2-GFP-infected MSCs (T-MSCs) or non-transfected MSCs (N-MSCs) were injected into the wound bed and margin of the excisional wound. These authors indicated that wounds in non-irradiated rats and irradiated rats took 17–18 days and 27–28 days, respectively, to heal. T-MSC administration and N-MSC administration were associated with a shorter healing time of 21 days and 24–25 days, respectively. MSCs promoted the deposition and remodeling of collagen in wounds. Significantly less bacterial colony formation was found in the cultured under-scar samples from the T-MSC administered wound bed. In our laboratory, when female B6D2F1 mice were exposed to 9.25 Gy of ^{60}Co gamma-ray (0.4 Gy/min) followed by a full-thickness excisional skin-wound (15% total body surface area). MSCs (3×10^6 cells) were intravenously injected. Their wounds were fully closed by day 21 after irradiation, whereas wounds in vehicle-treated irradiated mice were not fully healed yet at this time. Our results are in agreement with observations reported by other laboratories (Hao et al., 2009).

The benefit of MSCs can be a crucial factor in large animals as in small animals. Riccobono et al. (2012) reported that minipigs were locally irradiated at a dose of 50 Gy (^{60}Co gamma-ray) and wound healing was measured. These authors found that autologous adipocyte-derived MSCs improved cutaneous radiation syndrome wound healing, whereas allogeneic adipocyte-derived stem cells did not. In small animals, MSCs collected from different individuals seem not to be an issue (Németh et al., 2009). It is evident that MSCs prevent the rejection of allogeneic skin grafts (Xu et al., 2007).

15.3.8 MSCs Improve Radiation-Induced Cognitive Dysfunction

Radiotherapy frequently leads to progressive and long-lasting declines in cognition that can severely impact quality of life (Abayomi, 1996; Butler et al., 2006; Meyers and Brown, 2006). It has been reported recently that MSCs administration restores neuronal plasticity after irradiation. Acharya et al. (2015) reported that radiation on brain resulted in cognate dysfunction. When the brain of immunodeficient male athymic nude rats was irradiated at 10 Gy at 2.07 Gy/min, and then a total of 4×10^5 human neural stem cells (hNSC) were injected into four different sites of the hippocampus 2 days after irradiation, the hNSC transplantation promoted the long-term recovery of host hippocampal neurons and ameliorated cognitive function. The results are stunning and provide insights to further advance research in neuronal injury due to irradiation.

15.3.9 MSCs Improve Survival after Ionizing Radiation Combined Injury

Exposure to ionizing radiation alone or radiation combined with traumatic tissue injury (i.e., radiation combined injury, CI) is a crucial life-threatening factor in nuclear and radiological accidents. Radiation injuries occur at the molecular, cellular, tissue, and systemic levels. In our laboratory, we found that B6D2F1/J female mice exposed to ^{60}Co-γ-photon radiation (9.5 Gy, 0.4 Gy/min, bilateral) followed by 15% total-body-surface-area skin wounds (R-W CI) or burns (R-B CI) experienced a higher mortality over a 30-day observation period compared to irradiation alone (Kiang et al., 2010; Ledney and Elliott, 2010). Radiation combined injury was accompanied by severe leukocytopenia, thrombopenia, erythropenia, and anemia (Kiang et al., 2012).

This laboratory was the first one to investigate whether treatment with MSCs could improve survival after radiation combined injury. Bone marrow MSCs were isolated from femurs of B6D2F1/J female mice and were expanded and cultivated in hypoxic conditions (5% O_2, 10% CO_2, 85% N_2) over 30 days. MSCs ($2–3 \times 10^6$ cells/mouse) were transfused to mice 24 h after combined injury due to ^{60}Co-γ-photon irradiation (9.25 and 9.75 Gy, 0.4 Gy/min, bilateral) followed by skin wounding (i.e., radiation combined injury). Water consumption, body weight, wound healing, and survival tallies were monitored during observation period. Mice subjected to radiation combined injury experienced a dramatic moribundity over a 30-day observation period. Thus, radiation combined injury (9.25 Gy)-animal group was characterized by 40% mortality rate while radiation combined injury (9.75 Gy)-animal group had 100% mortality rate. Radiation combined

injury-induced sickness was accompanied by body weight loss, increased water intake, and delayed wound healing. At the 30th day post-injury, bone marrow cell depletion still remained in surviving radiation combined injury mice. Treatment of radiation combined injury (9.25 Gy)-animal group with MSCs led to an increase in 30-day survival rate by 30%, attenuated body weight loss, accelerated wound healing rate, and ameliorated bone-marrow cell depletion. Treatment of radiation combined injury (9.75 Gy)-animal group with MSCs led to an increase in 30-day survival rate by 20% (Kiang and Gorbunov, 2014), suggesting that MSC therapy is efficacious to sustain animal survival after radiation combined injury.

It is worthy to note that transfusion of MSCs with 1×10^6 cells failed to improve the survival after radiation combined injury. Transfusion of MSCs with more than 3×10^6 cells resulted in thrombosis and subsequent lethality in these mice. Thus, possibilities of multiple injections of MSCs less than 3×10^6 cells/mouse providing better desirable survival outcomes will warrant further investigation in this regard.

15.3.10 MSCs Attenuate the Severity of Acute Graft-Versus-Host Disease

Steroid and other immunosuppressants are standard treatments of graft-versus-host disease (GvHD). However, the possibility of lethal infections may still increase and no effective treatments are available for severe steroid-refractory GvHD (Salmasian et al., 2010). MSCs were targeted for treating the problem because of its possible immunosuppressive property. The latter has been controversial. Karlsson et al. (2008) reported in *ex vivo* human peripheral blood mononuclear cells (PBMCs) the effector function of virus-specific T-cells may be retained after MSC infusion. Kuzmina et al. (2012) also reported that *in vivo* infusion of $0.9–1.3 \times 10^6$/kg MSCs did not make difference in chronic GvHD development and infectious complications, although the mortality and occurrence of GvHD were reduced in MSC-infused patients. Le Blanc et al. (2008) reported that GvHD patients infused with single or multiple doses of $0.4–9 \times 10^6$ cells/kg bodyweight MSCs did not have side-effects during or immediately after infusions of MSCs. Furthermore, MSCs-infused GvHD patients had higher overall survival rate. The data were promising.

Animal models for GvHD have been available for studying the possibility of extending survival. Ren et al. (2008) reported that like human GvHD patients, infusion of 5×10^5 MSCs to GvHD-induced C57BL/6xC3H F1 mice on days 3 and 7 following bone marrow transplantation prevented GvHD induction. To use allogeneic MSCs instead of autologous MSCs, Yang et al. (2015) transfected MSCs with inducible co-stimulator (ICOS) gene that is a member of CD28 family and essential in T-cell activation and differentiation (Dong et al., 2001). Then, these allogeneic ICOS-transfected MSCs at 2×10^5 cells/mouse were injected intravenously into GvHD mice. The recipient mice were monitored daily for survival for up to 120 days. The gene transfected MSCs-injected mice survived better and were associated with lower incidence and severity of acute GvHD. These $MSCs^{ICOS}$ were able to suppress Th1 and Th17 polarization and promoted Th2 polarization on both protein expression and gene transcription levels. Higher serum levels of IL-4, IL-10, and lower levels of IFN-γ, IL-2, IL-12, and IL-17A were detected in $MSCs^{ICOS}$-injected mice. The results further reinforce that even allogeneic MSCs injection is also a promising strategy for acute GvHD prevention and treatment.

Animal models for GvHD have been available for elucidating the immunosuppression activity of MSCs. Ren et al. (2008) indicated that the immunosuppressive function of MSCs was elicited by IFNγ and the concurrent comitant presence of any of TNFα, IL-1α, or IL-1β. These combinations provoked the expression of several chemokines and iNOS in MSCs, thereby deriving T-cell migration into proximity with MSCs where T-cell responsiveness is suppressed by NO. Others laboratories reported that the MSC immunosuppressive function was involved in IL-10 (Batten et al., 2006), TGF-β (Groh et al., 2005), NO (Sato et al., 2007), indoleamine 2,3-dioxygenase (Meisel et al., 2004), and proglandin E2 (Aggarwal and Pittenger, 2005). However, Zangi et al. (2009) reported that MSCs are not intrinsically immune-privileged, and under allogenic settings, these cells induce rejection, which is followed by an immune memory. The long-term survival of allogenic MSCs likely represents a major challenge for use of allogenic MSCs for repair or regeneration of tissue and organs.

15.3.11 MSCs Preconditioned with Mood Stabilizers Enhances Therapeutic Efficacy for Stroke and Huntington's Disease

It is known that MSCs need to find their way to disease areas after being transplanted. Enhancing more MSCs to migrate and home to the relevant target regions may improve the overall therapeutic efficacy of MSC transplantation. Tsai et al. (2010) reported that MSCs treated with valproic acid and lithium, the mood stabilizers, increased MSC migration through histone deacetylases (HDACs), glycogen synthase kinase-3β (GSK-3β), and matrix metalloproteinase-9 (MMP-9). When these preconditioned MSCs (1×10^6 cells in 1 mL PBS, through the tail vein) were transplanted into male Sprague–Dawley rats 24 h after transient middle cerebral artery occlusion (MCAO), enhancement of MSC homing and migration was observed. Subsequently, MSCs reduced brain infarct volume, enhanced angiogenesis, and improved functional recovery (Tsai et al., 2011). When these preconditioned MSCs (3×10^5 cells in 12 μL PBS, intranasal injection) were injected into male and female N171-82Q Huntington's disease mice, MSCs reduced striatal neuronal loss and huntingtin aggregates and increased survival in these mice (Linares et al., 2016). These reports suggest that preconditioning stem cells with the mood stabilizers valproic acid and lithium before transplantation may be one of strategies to enhance MSC therapeutic efficacy.

15.4 Conclusions

MSCs have been extensively characterized. Promising experimental and clinical data are beginning to emerge to support the use of MSCs for repair or regeneration of tissues and organs. Therefore, MSCs as an effective therapy for patients/victims are promising. MSCs can be easily harvested from bone marrow and fat tissues. They can be cultured, grown, and expanded in the laboratory for mass production. Therefore, meeting commercial needs for health maintenance or tissue repair and regeneration can be envisioned and accomplished. With the advantage of autologous transplantation, which avoids immune rejection and ethical concerns, MSCs have great application prospects in personalized treatment of diseases.

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16

Stem Cell Therapeutics for Cardiovascular Diseases

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16.1 Introduction

Cardiovascular diseases (CVDs) are highly prevalent globally and produce immense health and economic burdens in the United States and throughout the world [1]. With age, physiological and pathophysiological alterations lead to compromised cardiovascular functions and elevated risk of CVDs, such as hypertension, atherosclerosis, or diabetes, even in the asymptomatic healthy elderly population [2]. In particular, tissue ischemia associated with coronary heart disease and peripheral arterial disease (PAD) accounts for the majority of all CVDs [1]. Although many CVDs are due to genetic defects, most can be attributed to unhealthy lifestyle factors, such as smoking, high salt, and/or high fat diet [1].

Recent advances in our understanding of the molecular mechanisms of CVDs have enabled major medical breakthroughs in diagnosis and treatment, such as drug treatment and organ transplantation; however, the fundamental pathogenic process of CVDs, that is, the loss of endothelial cells and cardiomyocytes, has not been fully understood and thus not satisfactorily resolved yet. The finding of stem cells and progenitor cells in circulating blood and vessel walls suggests that endogenous stem/progenitor cells possess the capacity to repair the damaged/injured endothelial cells, thereby maintaining the integrity of the vessel and, most importantly, restoring its function [3]. Over the last decade, cell-based therapy by stem or progenitor cells has emerged as a promising and potentially transformative approach for the treatment of various CVDs, such as myocardial infarction (MI), heart failure, or PAD [4]. Rapidly accumulating evidence has been documented to show that transplantation of various types of stem/progenitor cells, including bone marrow mononuclear cells (BM-MNCs), mesenchymal stem cells (MSCs), cardiac progenitor cells (CPCs), or endothelial progenitor

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cells (EPCs), effectively and efficiently replenish damaged cells, repair injured tissues, and restore/improve cardiac and vascular functions in many pre-clinical animal studies (summarized in Tables 16.1 and 16.2 later) and clinical trials (summarized in Table 16.3). Since endothelial dysfunction is one of the major problems for almost all CVDs, there is a growing interest and ongoing efforts to investigate EPCs and other stem/progenitor cell-derived endothelial cells as potential sources for cell therapy [5]. Therefore, in this chapter, we summarize the studies using EPCs and their derivatives to promote angiogenesis for the improvement of blood perfusion and tissue function in the treatment of ischemic cardiovascular diseases, mainly PAD and MI, in the preclinical and clinical settings.

16.2 Types of Stem/Progenitor Cell-Derived Endothelial Cells

In recent years, significant progress in stem cell research has been made, especially the mechanisms of stem cell activation, homing, and differentiation in promoting angiogenesis within ischemic cardiovascular diseases [6, 7]. Many studies have provided insights into the functional role of stem/progenitor cells in rescuing ischemic tissues (vascular regeneration) [8, 9] and the differentiation of stem/progenitor cells into vascular lineage [10–12]. There are a number of potentially therapeutic cell types, including pluripotent embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), adult bone marrow-derived mononuclear cells (BM-MNCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs), all of which have been demonstrated to differentiate into and contribute to endothelial repair and vascular regeneration [5, 13].

16.2.1 ESCs/iPSCs

Human embryonic stem cells (ESCs) are a valuable source of therapeutic endothelial cells due to their pluripotency, that is, they can differentiate into any type of cells found in the adult body [14]. They are also capable of infinite expansion with unlimited self-renewal ability. In certain culture conditions, ESCs are able to differentiate into vascular lineage *in vitro* and *in vivo* [15–20]. It was first described by Robert Langer's group who showed that human ESCs could differentiate into endothelial cells (ESC-ECs), forming vascular-like structures [15]. These ESC-ECs expressed endothelial cell markers, CD31, VE-cadherin, and von Willebrand factor (vWF), similar to human umbilical vein endothelial cells, and formed tube-like structures *in vitro* and microvessels *in vivo* [15]. Although there is no report yet for the use of ESC-ECs in human clinical trials, they have been shown to form vascular networks and restore blood perfusion in ischemic animal hind limbs *in vivo* [15, 16, 18, 19, 21] and improve cardiac function in animal models of ischemic heart disease [17, 20]. However, a few challenging factors limit the translational potential of ESCs in regenerative medicine, that is, the ethical concern of embryo destruction, the immunogenicity issue [22], and the potential risk of teratoma formation [23].

Another form of stem cells that can circumvent the aforementioned challenging issues and concerns with ESCs is induced pluripotent stem cells (iPSCs) that was originally reported by Yamanaka and colleagues who showed that somatic cells (i.e., fibroblasts) could be reprogrammed into a pluripotent stem cell state by viral transduction of four key transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) [24, 25]. It was later shown by the same group and others that these nuclear reprogramming-induced iPSCs can also be differentiated into endothelial cells (iPSC-ECs) [26–28]. These iPSC-ECs have been studied in preclinical models of ischemia and found to possess the capability of forming vascular networks and increasing blood perfusion in a murine model of hind limb ischemia [27] and promoting angiogenesis and cardiomyocyte survival and improving cardiac function in a murine model of myocardial infarction [28].

There is an increasing interest in the application of iPSCs in regenerative medicine because of several valuable feathers of these cells: (1) being autologous (no need of immunosuppression when delivered),

(2) pluripotency (can differentiate into almost all types of tissues), (3) non-controversy (are derived from adult tissue, not fetal tissue), and (4) rich source (can be derived from any adult cell type, such as skin fibroblast) [13]. Now new techniques are emerging that allow the generation of iPSCs via using non-genetic based approaches [29, 30], which will improve the safety of these cells for clinical use. In addition, the generation of patient-specific iPSCs by nuclear reprogramming of patient's own somatic cells via pluripotency genes provides an exciting alternative pluripotent stem cell source for their clinical use [31]. Thus, iPSCs may represent the most attractive patient-specific cell source for regenerative medicine.

16.2.2 MSCs

Mesenchymal stem cells (MSCs) are multipotent non-hematopoietic stem cells that possess the capacity for self-renewal and terminal differentiation into a variety of different cell types, including osteocytes, chondrocytes, adipocytes, hepatocytes, and smooth muscle cells [32, 33]. Of the various types of stem and progenitor cells, MSCs are being pursued the most actively in both preclinical and clinical investigations, due to their greater ease of isolation and capacity for *ex vivo* expansion. MSCs can home to and survive in an ischemic environment. Due to their paracrine, trans-differentiation, and immunosuppressive effects, they assist in the promotion of arteriogenesis and angiogenesis and terminally differentiate into vascular cells and myocytes by stimulation with growth factors, shear stress, and extracellular matrix complex [34–37]. These features enable MSCs to promote post-ischemic neovascularization and blood flow recovery in ischemic tissues associated with PAD and MI [38, 39]. Therefore, MSCs hold great promise as a therapy for ischemic vascular and heart diseases [33, 40, 41].

16.2.3 MNCs

Mononuclear cells (MNCs) from bone marrow and peripheral blood are the most extensively studied cell population for cell-based therapy. They are also the most commonly used therapeutic cells for clinical application of CVDs, due to the ease of isolation by density gradient centrifugation of bone marrow aspirates and peripheral blood and no need for *in vitro* expansion thus allowing for quick autologous application after harvest [42]. In addition, BM-MNCs and PB-MNCs represent a mixture of heterogeneous cell populations composed of several types of stem and progenitor cells including hematopoietic stem cells (HSCs), and non-HSCs such as MSCs, and EPCs. All these cells have been reported to either possess the ability to differentiate into vascular and/or cardiac cell types, or to provide paracrine factors supporting the repair or regeneration processes of injured tissues [43].

16.2.4 EPCs

In 1997, Asahara and colleagues first described the existence of circulating cells with endothelial lineage potential, that is, putative "endothelial progenitor cells" (EPCs) in the postnatal settings derived from MNCs that were isolated from human peripheral blood [44]. These so-called CD34+ EPCs or angioblasts could be differentiated into endothelium-like cells *in vitro* and to incorporate into neovasculature in animal models of ischemia [44]. Since then, increasing number of reports have been documented to examine the exact identity and therapeutic potential of EPCs in both preclinical and clinical studies.

In general, EPCs can be roughly defined as a group of cells that retain endothelial potential in the developmental pathway from hemangioblasts to fully differentiated endothelial cells. Although there is still a lack of unambiguous and consistent definitions of EPCs, they are generally characterized by positive expression of VEGFR2/Flk1, CD133/AC133, and CD34 (all are also cell-surface antigen markers expressed by HSCs) at early stages once attached onto tissue culture dishes [45, 46]. As EPCs mature toward endothelial cells in

expansion culture for several weeks, they start to express endothelial markers such as VE-cadherin, vWF, endothelial nitric oxide synthase (eNOS), and so on [46]. To date, however, no single marker or combination of markers identifies a pure EPC population. EPCs home to sites of tissue injury and participate in injury repair via directly incorporating into and forming new vessels and also indirectly secreting pro-angiogenic cytokines and growth factors [47].

16.3 EPC and Other Stem/Progenitor Cell Therapy in CVDs

16.3.1 EPC Therapy for Ischemic Vascular Diseases (PAD/HLI)

Peripheral artery disease (PAD), also known as peripheral vascular disease, is most commonly referred to as the ischemia of the limbs secondary to atherosclerotic occlusion [48]. The age-adjusted prevalence of PAD has been estimated as about 12% in the US population in 1985 [49]. PAD results from narrowing of the peripheral arteries that supply oxygenated blood and nutrients to the legs and feet. PAD most frequently presents with pain with walking, which is known as "intermittent claudication" [48]. Critical limb ischemia (CLI) is a chronic condition and the most severe clinical manifestation of PAD affecting a limb, defined as pain at rest due to reduced blood flow to the limb, which may further result in painful ischemic ulcerations and even limb-threatening gangrene [48].

Among preclinical models of PAD, the most popular and widely used animal model is the hindlimb ischemia model (HLI), which has been developed in mice, rats, rabbits, or even pigs [50, 51]. In this model, surgical ligation of the femoral artery at a specific site reduces blood flow to the lower leg by about 20% and thus triggers arteriogenesis of small, pre-existing collateral arteries into functional conduit vessels proximally and promotes ischemic angiogenesis distally [50, 51]. The HLI model has been widely used in evaluating the therapeutic capacity of stem/progenitor cells in various preclinical studies, due to its ease of access to the femoral artery and low mortality rate. Table 16.1 summarizes the preclinical animal studies of EPC cell therapy for PAD.

16.3.2 EPC Therapy for Ischemic Cardiac Diseases (MI)

Cell therapy has also emerged as a promising option to treat myocardial infarction or heart failure. The treatment of acute and chronic MI with stem and progenitor cells has been shown to produce a modest benefit in most but not all studies. Table 16.2 summarizes the findings of EPC cell therapy for MI in preclinical animal studies.

16.3.3 EPC Therapy in Clinical Trials for CVDs

Table 16.3 summarizes the clinical trials of EPC cell therapy for CVDs (including MI and PAD). Many clinical trials have investigated the safety and efficacy of EPCs in the treatment of CVDs. These studies have ranged from case reports to small, randomized, placebo-controlled trials, as outlined in Table 16.3.

16.4 Strategies and Approaches for Enhancing EPC Therapy in CVDs

Although EPC-based cell therapy has been well documented to improve the symptoms in patients with ischemic cardiac and/or vascular diseases, their limited plasticity and decreased number and/or function in patients with existing cardiovascular disease or smokers potentially limit the full benefit of autologous EPC therapy for cardiac and vascular regenerative medicine. Increasing number of studies have reported the

Table 16.1 *Stem cell/EPC therapy in pre-clinical (animal) studies of PAD*

Disease Models	Cell Types	Delivery Approaches	Follow-up Periods	Outcomes	Ref
Mouse / Rabbit HLI	Human CD34+; Mouse Flk-1+	Tail vein injection	1, 4, 6 weeks	Incorporated into active angiogenesis sites	[44]
Mouse HLI	Mouse BM-EPCs	Intravenous injection	1–4 weeks	Incorporated into capillaries among skeletal myocytes	[52]
Mouse HLI	Human EPCs	Intracardiac injection	3 days-4 weeks	Improved blood flow recovery and capillary density, reduced limb loss rate	[53]
Rat HLI (nude rats)	Human UCB and PB CD34+ MNCs (EPCs)	Intramuscular injection	2 weeks	Cells survived and participated in capillary networks, augmented neovascularization and blood flow in ischemic hindlimb	[54]
Mouse HLI (diabetic nude mice)	Human PB-CD34+ cells	Intramuscular injection	1–18 days	Accelerated blood flow restoration in diabetic mice, but not in nondiabetic mice	[55]
Rabbit HLI	Rabbit BM-MNCs	Intramuscular injection	2–4 weeks	More angiographically detectable collateral vessels, higher capillary density, greater blood perfusion	[56]
Mouse HLI (nude mice)	Human EPCs (VEGF-transduced)	Tail vein injection	1–4 weeks	Improved neovascularization and blood flow recovery, reduced limb necrosis and autoamputation, enhanced EPC incorporation	[57]
Mouse HLI (nude mice)	Human PB-EPCs (simultaneously with SDF-1)	Intramuscular SDF-1 and intravenous EPC injection	Day 3, 1– 4 weeks	Increased local accumulation of EPCs in ischemic muscle; improved ischemic tissue perfusion and increased capillary density.	[58]
Mouse HLI	Mouse MSCs	Intramuscular injection	4 weeks	Enhanced collateral flow recovery and remodeling, improved limb function, reduced autoamputation incidence, and attenuated muscle atrophy	[34]
Mouse HLI (SCID mice)	Human UCB-CD34+ / KDR+	Intramuscular injection	3 weeks	Improved limb salvage and hemodynamic recovery, superior neovascularization, attenuated endothelial cell apoptosis and interstitial fibrosis	[59]
Mouse HLI (nude mice)	Human UCB-CD133+ / VEGFR2+	Tail vein injection	3 days – 3 weeks	Incorporated into capillary networks in ischemic hindlimb, augmented neovascularization, and improved ischemic limb salvage	[60]
Rat HLI	Rat MSCs and BM-MNCs	Intramuscular injection	3 weeks	Improved blood perfusion, higher capillary density	[38]
Rat HLI	Human PB-derived CD133+ progenitor cells	Intramuscular injection (within collagen-based matrix)	2 weeks	Greater retention of CD133+ cells when delivered by matrix, incorporated into vascular structures, greater intramuscular arteriole and capillary density	[61]

(*Continued*)

Table 16.1 *(Continued)*

Disease Models	Cell Types	Delivery Approaches	Follow-up Periods	Outcomes	Ref
Mouse HLI (nude mice)	Human ESC-ECs (vWF+)	Intramuscular injection	2–4 weeks	Increased limb salvage, blood perfusion ratio, and capillary and arteriole densities; transplanted hESC-ECs localized as capillaries near muscle tissues in ischemic regions or incorporated in the vessels between muscle tissues	[16]
Mouse HLI (nude mice)	Human EPCs and SMPCs	Intravenous injection	2 weeks	Increased vessel density and foot perfusion, enhanced capillary and arteriolar densities	[62]
Mouse HLI (nude mice)	Human EPCs/OECs	Intramuscular injection of cell-loaded macroporous alginate scaffolds	2–6 weeks	Improved engraftment of transplanted cells in ischemic murine hindlimb musculature, increased blood vessel densities, improved cell efficacy in salvaging ischemic murine limbs, returned perfusion to normal levels, prevented toe and foot necrosis	[63]
Mouse HLI	Human ESC-ECs and ESC-mural cells	Intra-femoral artery injection	1–6 weeks	Improved blood flow recovery and capillary density, effectively incorporated into host circulating vessels	[18]
Mouse HLI	Mouse BM-MNCs or PB-MNCs	Intramuscular injection	4 weeks	Increased blood flow ratio, increased capillary density	[64]
Mouse HLI	Mouse ESCs or ESC-ECs	Intramuscular, intrafemoral artery or vein injections	2 weeks	ESC-ECs delivered by all 3 modalities localized to ischemic limb, engraftment of ESC-ECs into the limb vasculature, ESC-ECs significantly improved limb perfusion and neovascularization	[19]
Mouse HLI	Human iPSC-ECs	Intramuscular injection at day 0 and day 7	2 weeks	Increased blood perfusion ratio, greater total number of capillaries	[27]
Mouse HLI	Human HUVECs and UBC-MSCs	Intramuscular injection of 3D HUVECs /UCB-MSCs aggregates	2 weeks	Recovered blood perfusion, enhanced functional vessel formation, protected ischemic limb from degeneration	[65]
Mouse HLI (SCID mice)	Human MNCs-ECs, ESC-ECs, iPSC-ECs	Intramuscular injection	1–4 weeks	Attenuated severe hind-limb ischemia in mice via enhancement of neovascularization	[66]
Mouse HLI (nude mice)	Human AFSC-ECs	Intramuscular injection	4 weeks	Increased limb salvage, improved ischemic/normal limb blood perfusion ratio, increased capillary and arteriole densities, transplanted cells incorporated into vessels in ischemic region	[67]
Mouse HLI (diabetic nude mice)	Human WJ-EPCs: EPCs isolated from Wharton's jelly of umbilical cord	Intramuscular injection	3 days, 1 week	Restored blood flow and function, improved muscular morphology, stimulated neovascularization, and decreased cell apoptosis in ischemic hind limbs of diabetic mice	[68]

Abbreviations: UCB: umbilical cord blood; PB: peripheral blood; SMPCs: smooth muscle progenitor cells; OECs: outgrowth endothelial cells; HUVECs: human umbilical vein endothelial cells; AFSCs: Amniotic fluid-derived stem cells; WJ-EPCs: EPCs isolated from Wharton's jelly of the umbilical cord; IGF-1: insulin-like growth factor-1

Table 16.2 *Stem cell/EPC therapy in pre-clinical (animal) studies of MI*

Disease Models	Cell Types	Delivery Approaches	Follow-up Periods	Outcomes	Ref
Mouse MI	Mouse BM-EPCs	Intravenous injection	1–4 weeks	EPC incorporation into neovascularization foci at infarct border	[52]
Mouse MI	Mouse Lin- c-kit + BM cells	Intramyocardial injection in contracting wall bordering the infarct	9 days	*De novo* myocardium generation comprising proliferating myocytes and vascular structure, newly formed myocardium occupying 68% infarcted portion	[69]
Rat MI (nude rats)	Human PB-EPCs	Intravenous injection	1–4 weeks	Transplanted EPCs accumulated in ischemic area and incorporated into foci of myocardial neovascularization; echocardiography revealed smaller ventricular dimensions and greater fractional shortening, better preserved regional wall motion, greater capillary density, less left ventricular scarring	[70]
Rat MI (nude rats)	Human PB-CD34+ cells	Tail vein injection	2–15 weeks	Decreased apoptosis of hypertrophied myocytes in the peri-infarct region, long-term salvage and survival of viable myocardium, reduction in collagen deposition and sustained improvement in cardiac function	[71]
Pig MI	Pig BM-MNCs or PB-MNCs	Trans-endocardial injection	4 weeks	Increase in systolic function and regional blood flow, reduction of the ischemic area, increase in collateral vessel formation and capillary numbers	[72]
Pig MI	Pig MSCs	Intramyocardial injection	2–4 weeks	Robust engraftment of MSCs, expression of muscle-specific proteins, attenuated degree of contractile dysfunction, reduced extent of wall thinning after MI	[73]
Rat MI	Rat MSCs (Akt1-engineered)	Intramyocardial injection	2 weeks	Inhibited cardiac remodeling by reducing intramyocardial inflammation, collagen deposition and cardiac myocyte hypertrophy, regenerated 80–90% of lost myocardial volume, and normalized systolic and diastolic cardiac function, greater myocardial volume	[74]
Rat MI (nude rats)	Human PB-CD34+ angioblasts (EPCs)	Tail vein injection	2 weeks	Dose-dependent neovascularization with development of larger-sized capillaries; sustained improvement in cardiac function by mechanisms involving protection against apoptosis and induction of proliferation/regeneration of endogenous cardiomyocytes	[75]

(*Continued*)

Table 16.2 *(Continued)*

Disease Models	Cell Types	Delivery Approaches	Follow-up Periods	Outcomes	Ref
Pig MI	Pig MSCs	Intravenous, intracoronary, or endocardial delivery	14 days	Greater engraftment within infarcted tissue by intracoronary and endocardial injection than intravenous infusion	[76]
Rat MI	Rat MSCs	Monolayered cell graft placed on the surface of the anterior scar	4 weeks after cell transplantation (8 weeks after MI)	Engrafted sheet formed a thick stratum that included newly formed vessels, undifferentiated cells and few cardiomyocytes. MSC sheet also acted through paracrine pathways to trigger angiogenesis. monolayered MSCs reversed wall thinning in scar area and improved cardiac function in rats with MI	[77]
Rat MI (extensive MI) nude rats	Human UCB-CD133+	Intravenous infusion	1 month	Improved LV fractional shortening, transplanted cells migrated, colonized, and survived in infarcted myocardium, and incorporated into endothelial cells, scar tissue significantly populated with autologous myofibroblasts	[78]
Mouse MI	Mouse ESC-ECs	Intramyocardial injection	8 weeks	Longitudinal survival of transplanted ESC-ECs, significant functional improvement, increased presence of small capillaries and venules in the infarcted zones	[17]
Pig MI (mini-swine)	Pig CD34+	Intracoronary injection	2–6 weeks	Greater cardiac repair, better collateral vessels, host vascular niche contributing to myocardial repair	[79]
Rat MI (Sprague-Dawley rats)	Human PB-EPCs	Injected into border infarct zone	8 weeks	Improved fractional shortening, increase in left ventricular developing pressure, elevated coronary flow rates, increased density of CD31+ vessel structures indicating higher rate of neovascularization, reduced amount of apoptotic cells and inflammatory cells	[80]
Mouse MI (NOD/SCID mice)	Human myoendothelial cells (CD34+ /CD56+ / CD144+)	Intramyocardial injection	5 days, 2–6 weeks	Greater improvement in left ventricular function, robust engraftments within the infarcted myocardium, stimulated angiogenesis, attenuation of scar tissue, more effective proliferation and survival of endogenous cardiomyocytes	[81]
Rat MI (Lewis rats)	Tissue-engineered ECM scaffold seeded with EPCs primed with SDF-1	Sutured to the anterolateral left ventricular wall	4 weeks	Increased VEGF levels, increased vessel density, enhanced microvascular perfusion, improved vasculogenic response, preserved left ventricular internal diameter, decreased infarct scar formation	[82]

Rat MI (Sprague–Dawley rats)	Rat PB-EPCs, transduced with IGF-1	Intramyocardial injection	12 weeks	Improved cardiac function, reduced cardiac apoptosis, increased cardiomyocyte proliferation, increased numbers of capillaries	[83]
Pig MI Mouse MI	Human ESC-ECs and human ESC-SMCs	Fibrin-cell patch applied to the LV anterior wall of the MI area	1 and 4 weeks	MRI revealed significant LV functional improvement, significant engraftment of hESC-derived cells, increased neovascularization	[20]
Mouse MI	Mouse Lin- Sca1+ CD31+ EPCs, and human CD34+ cells, treated with inhibitors	Intramyocardial transplantation	1–4 weeks	Improvement in ventricular functions, *de novo* cardiomyocyte differentiation, increased capillary density and reduced fibrosis	[84]
Mouse MI	Pig iPSC-ECs	Intramyocardial injection	4 weeks	Functional improvement measured by echocardiography, promoted proangiogenic and antiapoptotic cytokine release *in vitro* and *in vivo*	[28]
Pig MI (mini-swine)	Pig PB-EPCs	Intramuscular injection into the peri-infarcted regions	1 day – 4 weeks	Impeded heart failure development, preserved left ventricular function and dimensions, inhibited infarct expansion	[85]
Pig MI	Human iPSCs	Catheter-based intramyocardial injection	15 weeks	hiPSCs could be visualized for up to 15 weeks, hiPSC-derived endothelial cells contributed to vascularization.	[86]

Table 16.3 *Stem/progenitor cell/EPC therapy in clinical studies of CVD*

Trial Design and Patients	Disease Types	Cell Types	Delivery Approaches	Follow-up Periods	Outcomes	Ref
25 patients (unilateral ischemia); 22 patients (bilateral ischemia); within-patient controls	CLI	Autologous implantation of BM-MNCs, PB-MNCs	Intramuscular injection	4–24 weeks	Improved ankle-brachial index (ABI) in legs injected with BM-MNCs than in PB-MNCs or saline; improvements for transcutaneous oxygen pressure, rest pain, and pain-free walking time	[87]
7 patients, no controls	CLI	Autologous BM-MNCs	Intramuscular injection	4–24 weeks	Improved ankle-brachial pressure index, transcutaneous oxygen pressure, and pain-free walking time; enhanced leg blood flow response to ACh	[88]
6 patients; no control	AMI	PB-CD34+ cells	Intracoronary injection	4 months	No restenosis or arrhythmia, enhancement in wall motion score index, myocardial homing of transplanted cells	[89]
44 cell-injected patients, 22 control patients	AMI	BM-MNCs	Intracoronary injection	3 months	Increased baseline peak systolic velocities of longitudinal contraction of the infarcted wall; Increased baseline left ventricular ejection fractions	[90]
15 patients, no controls	severe ischemic PAD	autologous PB-MNCs	Intramuscular injection	4 to 16 months (mean 9.3 months)	Improved ankle-brachial index values, walking distance, pain scale, and transcutaneous oxygen pressure	[64]
41 cell-injected patients, 45 saline-injected patients	ST-segment elevation MI	autologous BM-MNCs	Intracoronary injection	4 years	Improved left ventricular ejection fraction; however, no improvement of myocardial viability of infarcted area assessed by SPECT	[91]
7 patients; 5 nonrandomized control patients	Anterior MI	PB-CD34+ cells	Transcoronary, intracoronary infusion	3 months	Decrease in end-systolic volume, myocardial homing of infused cells	[92]
11 patients (EPCs); 29 patients (BMSCs)	End-stage ischemic or dilated cardiomyopathy	PB-CD34+ (EPCs); BMSCs	Epicardial, intracoronary artery, and intrapulmonary artery injections	3–12 months	Marginal improvement in myocardial function (increase in ejection fraction)	[93]
Phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial; 17 patients, no control	Atherosclerotic PAD or Buerger's disease with CLI	PB-CD34+ cells	Intramuscular injection	12 weeks	Improvement in limb ischemia, improved pain rating scale, TBPI, transcutaneous partial oxygen pressure, total or pain-free walking distance, and ulcer size, no death or major amputation	[94]

7 patients; no control	AMI	PB-CD34+ cells	Intracardiac delivery	24–76 months (average 49 months)	Improvement of heart function parameters, increased left ventricular ejection fraction values, myocardial structure regeneration and revascularization	[95]
7 patients; no control (2 patients died due to noncardiac conditions)	Chronic post-infarct heart failure	PB-CD133+ cells	Intracoronary infusion (intracoronary percutaneous angiography)	24 months	Improved NYHA scale, decreased plasma pro-B-natriuretic peptide and sudden death risk, increased ejection fractions	[96]
28 patients, no controls	CLI	CD34+ CD133+ EPCs	Intramuscular injection	1–48 months (mean 14 months)	No adverse effects observed, decrease in pain scale, increased limb salvage rate	[97]
Randomized, double-blinded, placebo-controlled trial; 25 cell-injected patients, 25 placebo solution-injected patients	Chronic myocardial ischemia	Autologous BM-MNCs	Intramyocardial injection	3 months	Increased left ventricular ejection fraction; improved Filling pressure estimate E/E′ ratio; larger improvement in E/E′ ratio; increased E/A peak flow ratio as assessed by MRI	[98]
Phase II, prospective, double-blind, randomized, 112 cell-treated patients; 56 placebo-treated control patients	Refractory angina	Autologous CD34+ cells	Intramyocardial injection	12 months	Lower weekly angina frequency, greater improvement in exercise tolerance, 5.4% mortality in Placebo-treatment group with no deaths among cell-treated patients	[99]
Phase III, randomized, double-blind, placebo controlled, multicenter trial; 71 cell-treated patients; 71 placebo-treated control patients	MI	Autologous CD133+ cells	Intramyocardial injection	6 months	Higher LV ejection fraction	[100]
Phase I/IIa clinical trial; 17 patients; no control	CLI	Autologous, GCSF-mobilized CD34+ cells	Intramuscular transplantation	208 weeks (4 years)	Improvement of toe brachial pressure index and transcutaneous partial oxygen pressure, improved pain rating scale, ulcer size, and exercise tolerance	[101]

(*Continued*)

Table 16.3 *(Continued)*

Trial Design and Patients	Disease Types	Cell Types	Delivery Approaches	Follow-up Periods	Outcomes	Ref
25 patients, no controls	CLI	PB-CD34+ cells (G-CSF mobilized)	Intramuscular injection	6 to 33 months	Decreased pain rating scale score, increased peak pain-free walking time, increased ankle-brachial index, increased transcutaneous partial oxygen pressure	[102]
5 patients, no controls	Chronic ischemic cardiomyopathy	Autologous CD133+ cells	Intramyocardial injection	18 months	Increased systolic wall thickening, improved mean segmental wall thickness	[103]
Phase II, single-arm, non-randomized study; 11 patients (1 patient discontinued due to amputation), no controls	CLI	PB-CD34+ cells (G-CSF mobilized)	Intramuscular injection	52 weeks	Improved ischemic rest pain scales; improved perfusion pressure, transcutaneous partial oxygen pressure, pain-free walking distance, total walking distance, and toe brachial pressure index; improved Rutherford's category; increased CLI-free ratio	[104]
49 patients, no controls	CLI	Autologous BM-MNCs	Dual intramuscular and intra-arterial injections	Mean 18.4 months	Delayed or prevented major limb amputations, improved ABI measurements, relieved rest pain, and improved ulcer healing	[105]

Abbreviations: AMI: acute myocardial infarction; BMSC: mesenchymal bone marrow stem cells; SPECT: single-photon emission computed tomography

development and use of different strategies to enhance the efficacy and potential of EPC therapy via genetic or non-genetic approaches [106]. The modifiers or key factors that have been shown to enhance stem/progenitor cell therapeutics in CVDs include, but are not limited to, chemokine receptors such as CXCR4 [107–110], CXCR2 [111–114], CX3CR1 [113], CXCR7 [115–117], and CCR2 and 5 [118–120], growth factors and their cognate receptors such as VEGF1/2/3 [57, 121, 122], PDGF [123, 124], FGF-1/2 [124–128], IGF-1/2 [83, 129–131], and HGF [131], and so on. Other signaling molecules or factors that have been demonstrated to promote EPC survival and function are eNOS/nitric oxide (NO) [132–136], AMP-activated protein kinase (AMPK) [137–140], protein kinase B (PKB/Akt) [122, 141], heme-oxygenase-1 (HO-1) [139–142], manganese superoxide dismutase (MnSOD) [137, 143], erythropoietin (EPO) [144–146], peroxisome proliferator-activated receptor (PPAR) agonists [147–151], statins [134, 152, 153], and so on. Therefore, *ex vivo* modification of EPC cellular function prior to transplantation might harbor specific benefits for patients with impaired EPC functions.

An increasing number of studies have been documented to examine the promoting effects of these molecules or key factors on rescuing defective functions of EPCs from diabetic patients or aged people. For example, gene modification of human EPCs through *ex vivo* transduction of Akt/HO-1, two intracellular proteins that play an important role in angiogenesis and cell survival, has been shown to significantly enhance myocardial infarction recovery in nude mice [141]. It has been report that decreased expression of MnSOD in EPCs contributes to impaired would healing in a mouse model of type 2 diabetes [143]. However, transplantation of diabetic EPCs after ex vivo MnSOD gene transfer restored their ability to promote angiogenesis and wound repair [143]. The same group later on showed that activation of AMPK, a serine/threonine protein kinase, rescues impaired EPC functions in diabetes and suppresses mitochondrial superoxide by inducing MnSOD [137]. In view of an important role of eNOS in angiogenesis, many studies evaluated the effects of eNOS gene transfer in EPC function. Kaur *et al* demonstrated that genetic engineering with eNOS in *ex vivo* expanded EPCs isolated from patients with coronary artery disease potently improved EPC functional properties, such as proliferation, migration, differentiation and adhesion/integration into tube-like structures *in vitro* [136]. In addition, transplantation of EPCs overexpressing eNOS also repairs the injured vessel by inhibiting neointimal hyperplasia and restoring vascular function in balloon injury rat model [135] and rabbit model [142]. Therefore, eNOS gene transfer is a valuable approach to augment angiogenic properties of *ex vivo* expanded EPCs and eNOS-modified EPCs may offer significant advantages than EPCs alone in terms of their clinical use in patients with CVDs. A variety of chemokine receptors and their cognate ligands have also been reported to promote EPC survival and angiogenic function. SDF-1/CXCR4 signaling has been extensively reported to play an important role in EPC mobilization and angiogenesis [107–109]. Chen *et al* demonstrated that transplantation of CXCR4-primed (over-expressed) EPCs reduces cerebral ischemic damage and promotes repair in diabetic mice, via enhancing EPC colonic forming capacity and EPC infusion efficacy, and preventing EPCs from high glucose-induced dysfunction [154]. Recently, our group has shown that CXCR2 macromolecular signaling complex plays an essential role in mediating EPC functions and angiogenesis *in vitro* and *in vivo* [114]. Therefore, genetic modification of EPCs to overexpress angiogenic growth factors or chemokine receptors, enhance angiogenic signaling responses, rejuvenate the bioactivity, and/or extend the life span of EPCs represents a potential approach that might address the limitations of EPC transplantation (i.e., quantitative and qualitative impairment) and thereby optimize therapeutic neo-vascularization.

16.5 Concluding Remarks

Stem/progenitor cell therapy has a great potential to address a critical need in restoring damaged heart and vessels. The involvement of EPCs and other types of stem/progenitor cells in postnatal vasculogenesis, arteriogenesis, and endothelial repair is supported by growing preclinical evidence. Current clinical studies also

support a small but significant benefit of autologous cell transplantation with respect to cardiac function and blood perfusion in the settings of both MI and PAD. However, there are still numerous obstacles that remain to be overcome or resolved before widespread application of EPCs in the treatment of CVDs [155, 156].

Effective cell characterization, isolation, and processing methods must continue to be refined in order to obtain a better understanding of the feature that defines potency. For instance, both the cell origin and surface markers of EPCs still remain controversial [46, 155, 157]. Therefore, further studies are required to identify a standardized method to define EPCs, probably through lineage tracing and functional analysis [158]. Cell delivery method, timing, and dosing regimens are also needed to be tailored to the disease state and clinical trajectory in order to achieve optimal outcomes [76, 159]. Furthermore, survival of transplanted cells in the hypoxic, ischemic/necrotic, and immunoreactive host environment is still a challenge in practice [22]. Thus, adjunctive therapies (such as by drug statins or lifestyle change) to overcome endogenous impairments in EPC health and vascular responsiveness must also be developed, along with molecular and bioengineering tools to augment therapeutic effects of infused cells in time and space [160]. The safety of stem/progenitor cell-based treatment approaches also need to be closely monitored with respect to the occurrence of adverse events, such as teratoma formation, especially in the case of pluripotent stem cells [23].

In summary, although EPC-based therapy is still in its early stage, the studies from past preclinical and clinical investigations have provided a wealth of information for further refinement of stem/progenitor cell therapy. With more efforts to understand the molecular mechanisms of stem cell function and continued commitment to rigorous, randomized, placebo-controlled clinical trials, stem cell regimen may represent an integral part of routine regenerative therapy for treating CVDs and may also shift the paradigm of cardiovascular care.

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17

Stem-Cell-Based Therapies for Vascular Regeneration in Peripheral Artery Diseases

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Peripheral arterial disease (PAD) includes non-occlusive arterial disease, such as aneurysms, traumatic, or congenital arteriovenous fistulas as well as all non-coronary arterial occlusive diseases. Typically, arterial occlusion results from arteriosclerosis but can also originate from pathobiology intrinsic to the vessel wall, as in vasculitis, thromboangiitis obliterans, or fibromuscular dysplasia; or secondary to extrinsic pathology as in compression syndromes, such as popliteal artery entrapment. Atheroembolism from a diseased aorta or dissection of the aorta may also cause obstruction of peripheral arteries[1]. Among the different forms of PAD, atherosclerosis of the abdominal aorta, iliac, and lower-extremity arteries is a major health problem that is, however, undertreated and poorly understood by the medical community[2]. Although it is frequently under- or un-diagnosed, it has been estimated that 4.3% of the United States population of 40 years and older has lower extremity PAD[3]. A similar prevalence of PAD has been found in Western Europe, with population studies estimating the prevalence between 4–8%[4]. Patients with PAD display high risk of morbidity, including stroke and myocardial infarction, and the mortality rate reaches 50% within 6–10 years following the diagnosis. Intermittent claudication, defined as pain in the muscles of the leg with ambulation, is the earliest and the most frequently presenting symptom in patients with lower extremity PAD. As the disease progresses in severity, patients may have pain at rest. Although claudication symptoms are typically localized in the calf or the thigh, "rest pain" is characteristically in the foot. In the late stages of PAD, tissue hypo-perfusion progresses to ischemic ulceration and gangrene, and major amputation is eventually required in more than a third of these patients. Such final stage of progression is defined as critical ischemia.

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A variety of risk factors have been identified for PAD. As such, preventable or treatable risk factors for PAD are generally thought to mirror other forms of cardiovascular disease and include cigarette smoking, type 2 diabetes, and clinically elevated levels of blood pressure and cholesterol, which are the main therapeutic targets in clinical and prevention guidelines[5]. Consistent with this, in a recent large prospective cohort study, the combination of the four clinical risk factors of smoking, hypertension, hypercholesterolemia, and type 2 diabetes was strongly and independently associated with risk of confirmed and clinically significant PAD. Duration of diabetes and hypercholesterolemia, severity of hypertension, and cumulative intensity of smoking all demonstrated graded relationships with risk[6].

When the disease advances to the later stage of critical ischemia, the restoration of perfusion by bypass surgery or angioplasty should be considered as a first-line treatment. However, such treatment is possible in only 70% of cases. Furthermore, the durability of bypasses, particularly in cases of critical ischemia, is poor and is no more than 50–70% 3 years after surgery. No other treatment has proved effective in this context. Current treatment is thus based on local care of trophic problems, possibly combined with systemic antibiotic treatment. Such ultimate failure of medical treatment and procedural revascularization in significant numbers of patients paves the way for the development of alternative therapies for PAD targeting the vascular compartment. Indeed, arterial occlusion leads to profound tissue hypo-perfusion and alteration of the metabolic homeostasis. Of interest, when the vascular response to metabolic tissue demands is chronically insufficient, structural alterations in the vasculature are initiated to counteract the local reduction in partial oxygen pressure. These structural alterations include vasculogenesis; that is, the incorporation of stem/progenitor cells into the regenerating microvasculature; angiogenesis, that is, sprouting of endothelial cells (ECs) from pre-existing capillaries, followed by their proliferation, migration, and capillary formation; arteriogenesis, that is, formation of new arterioles from pre-existing capillaries; and collateral growth, that is, the remodeling of existing collateral channels, so that they can deliver more blood flow to the limb[7].

Hence, cell-free or cell-based strategies have been developed to tackle such structural vascular alterations and increase the number of vessels in the affected territories in order to improve tissue perfusion[7]. These approaches include administration of angiogenic factors, either as recombinant protein or as gene therapy, and more recently, lead to investigations of stem/progenitor cell therapy. The purpose of this chapter is to provide an overview of the preclinical basis for stem cell therapies, and to summarize the clinical research that has been done, mainly in patients with atherosclerotic arterial occlusive disease and thromboangiitis obliterans.

17.1 Sources of Stem Cells for Vascular Regeneration

17.1.1 Adult Stem Cells

Stem cell-based therapies for vascular regenerative medicine initially relied on a very simple concept: therapeutic stem/progenitor cells might differentiate into vascular cells, mainly of endothelial phenotype, increasing new vessel formation and tissue perfusion in the ischemic milieu. This exciting notion challenged the scientific community to start the quest for the Holy Grail in vascular regenerative medicine: the search for the ideal type of endothelial stem/progenitor cells. This concept premiered on seminal work from Isner's laboratory identifying a population of endothelial progenitor cells (EPCs) in human peripheral blood able to incorporate into sites of active angiogenesis[8]. These circulating EPCs originate from the bone marrow (BM) and are mobilized endogenously in response to tissue ischemia or exogenously by granulocyte macrophage-colony stimulating factor (GM-CSF) therapy[9,10]. Since EPCs are mobilized from the BM into the circulation, the classical method of EPCs isolation is the culture of selected/unselected peripheral blood mononuclear cells (MNCs) in specialized media, with subsequent isolation of two types of EPCs[11]. Early EPCs, which,

after 4–7 days of culture, have a weak proliferative potential and express leukocyte markers, such as CD14, CD45, and CD11b[12]. Late EPCs are adherent colonies that emerge after 1–3 weeks in culture and display a strong potential for proliferation. These cells express CD34 and the VEGF receptor-2. The differentiation potential of these cells depends on their origin, and this led the team of Ingram to establish a hierarchy of late EPCs, similar to the classification established for hematopoietic stem cells[13]. There is now a consensus that these late EPCs should be referred to as endothelial colony-forming cells (ECFCs). However, EPCs are imprecisely defined as any cell that retains endothelial potential in the developmental pathway from pluripotent stem cells to fully differentiated ECs. For example, hematopoietic stem cells and EPCs have been noted to share a variety of cell-surface antigen markers, namely CD34 and CD133. In addition, acquisition of a specific cellular phenotype is a dynamic process participating to the EPCs heterogeneity as well as to the failure in the development of suitable experimental approach to isolate and characterize such *bona-fide* EPCs. For example, as EPCs mature toward endothelial cells CD133 expression wanes, whereas adhesion molecule expression increases and endothelial functions such as low-density lipoprotein uptake and nitric oxide synthesis are gained. Finally, EPCs represent less than 1% of all BM cells and less than 0.01% of peripheral blood mononuclear cells. In addition, expansion and culturing of circulating EPCs are limited in adult individuals[13,14].

To overcome these limitations, many strategies of therapeutic revascularization have been established with cell populations thought to contain these BM–derived EPCs, mainly MNCs from the bone marrow or mobilized into the peripheral blood. Hence, BM-derived MNCs have been shown to promote vessel growth in experimental model of critical limb ischemia (CLI)[15–19]. As MNCs, harvested from either BM or blood, are comprised of a heterogeneous mix of hematopoietic cells and non-hematopoietic stromal cells such as mesenchymal stem cells (MSC), a variety of cell type from BM origin has been studied as a potential PAD treatment. Consistent with this, transplantation of CD34- or CD117- positive BM-derived cells using *in vivo* models of hindlimb ischemia demonstrated that intramuscular administration of marker-specific cells selected from the marrow augment ischemic tissue perfusion[20–22]. The therapeutic potential of BM derived MSC has also been extensively investigated. The minimum criteria for their characterization are: adhesion to plastic, expression of the surface markers CD73, CD90, and CD105, absence of the hematopoietic markers CD34, CD45, CD19, CD11a, and HLA-DR, and differentiation into osteoblasts, adipocytes, and chondrocytes *in vitro*. These cells have been shown to improve vessel density and tissue perfusion in experimental models of CLI[23–27].

Very small embryonic-like stem cells are another resident population of multipotent stem cells in the bone marrow that express CD133 and pluripotency markers including Oct-4 and Nanog. Very small embryonic-like stem cells triggered post-ischemic revascularization in immunodeficient mice and acquired an endothelial phenotype either *in vitro* when cultured in the presence of VEGF-B or *in vivo* in Matrigel implants[28,29].

Of note, in a parabiotic model, hindlimb ischemia led to the incorporation of circulating CD117+CD45- progenitors in the microvasculature of the ischemic limb. Bone marrow replacement studies revealed that about half of these cells were derived from the bone marrow and about half were not[30]. Hence, it is likely that numerous complex tissues, such as those of the heart, brain, intestine, liver, adipose tissue and the vascular wall contain reservoirs of adult stem/progenitor cells with vasculogenic potential[31–33]. The regeneration potential of these cells of different origins is variable and depends on multiple mechanisms, probably according to the type of adult stem cells and the nature of the ischemic tissue[30,31]. In particular, MSC can be isolated from adipose tissue[33,36], synovial tissue[37], lung tissue[38], umbilical cord blood[39], and even peripheral blood[40]. Adipose tissue is a particularly promising potential source of MSCs, because this tissue is abundant and easy to obtain, by liposuction. Two types of cells with vascular differentiation potential have been isolated from adipose tissue: adipose tissue-derived stromal cells (ADSCs) obtained after culture must be distinguished from the stromal vascular fraction (SVF) generated by digestion of the adipose tissue. ADSCs have several characteristics in common with bone marrow-derived MSCs. However, the two types of immature stromal cells in adipose tissue have been shown to be effective in experimental models of tissue ischemia[33,34,41,42].

Finally, stem progenitor cells could be localized into the vascular structure. A population of CD34-positive cells, located around the vasa vasorum in the adventitia of arteries and veins, expresses typical pericyte markers (NG2, PDGFRβ, and RGS5) together with mesenchymal (CD44, CD90, CD73, CD29) and stemness antigens (Oct-4 and Sox-2). CD34-positive progenitor cells from human fetal aorta adventitia possess a robust regenerative capacity in mouse models of peripheral limb ischemia and ischemic below-knee ulcers in diabetic mice[43,44]. Interestingly, a similar clonogenic population of CD34-positive/CD31 – negative progenitors have been isolated and cultured from saphenous vein leftovers of patients undergoing coronary artery bypass graft surgery[45] On transplantation into mouse ischemic limbs, saphenous vein-derived pericyte progenitors proved to be superior to an equal dosage of circulating proangiogenic cells in supporting blood flow recovery[45].

17.1.2 Umbilical Cord-Blood-Derived Stem Cells

Human umbilical cord blood (UCB) has been shown to contain a large number of hematopoietic colony-forming cells. UCB progenitor cells are routinely used in patients affected by major hematological disorders as an alternative to bone marrow transplantation for stem cell reconstitution. In contrast to adult BM–progenitor cells, cord blood progenitors have distinctive proliferative advantages, including the capacity to form a greater number of colonies, a higher cell-cycle rate, and a longer telomere. In addition, cord blood can be obtained noninvasively as compared to invasive bone marrow isolation. It has been shown that CD34-positive cells obtained from human UCB give rise to mature endothelial cells when cultured onto specific substrates or by stimulation with growth factors in culture. Furthermore, it has been described the ability of these cells to improve neovascularization and to increase blood flow in immunodeficient animal models of hindlimb ischemia[21,46]. Similarly, freshly isolated human UCB CD34+ cells injected into ischemic adductor muscles gave rise to endothelial and, unexpectedly, to skeletal muscle cells in mice. In fact, the treated limbs exhibited enhanced arteriole length density and regenerating muscle fiber density[47]. MNC or CD34-positive enriched cells purified from human UCB have also the potential to differentiate into spindle-shaped cells expressing α-smooth muscle actin, smooth muscle heavy chain and calponin. These cells also display a carbachol-induced contractility and are therefore of a smooth muscle cell phenotype. A tight cooperation between endothelial cells and smooth muscle cells/pericytes is critical for the development of functional neovessels. In addition, stabilization of neovessels regulates blood flow vascular permeability and also endothelial cell functions, such as proliferation, survival, and migration. As a result, co-administration of human UCB-derived EPC and smooth muscle cells progenitors (SMPCs) trigger post-ischemic revascularization in mice with CLI[48].

17.1.3 Embryonic Stem Cells

Human embryonic stem cells (hESCs) are totipotent cells derived *in vitro* from embryos in the early stages of development. ESCs can give rise to the three primordial cell layers: the endoderm, mesoderm, and ectoderm. They also have an almost unlimited capacity for replication. These two characteristics distinguish them very clearly from adult stem cells. ESCs can differentiate into vascular cells in experimental models based on embryoid bodies (three-dimensional culture) or two-dimensional differentiation in the presence of specific growth factors. However, stem cell progeny are only partially restricted to a subset of potential fates. So, it is unlikely that a single even highly competent and plastic pluripotent cell can go through all these cell fate choices, and at each crossroad take the unique and the right decision to become a vascular cell[49]. Moreover, the unbridled enthusiasm regarding the use of pluripotent hESC has been dampened by major safety concerns such as their ability to generate teratomas. In addition, a progenitor cell could stop in the course of differentiation and proliferate in an uncontrolled manner *in vivo*, as reported with neuronal progenitors grafted

in rat brain. Thus, various cell culture techniques have been developed to obtain specific EPC from hESC (hESC-EPC)[50] with several properties in common with endothelial lineage: the expression of surface markers (e.g., CD31), the presence of intracellular proteins (e.g., von Willebrand's factor and endothelial nitric oxide synthase) and functional capacities (e.g., the formation of endothelial tubes in Matrigel)[51,52]. The potential of hESC-EPC to promote revascularization have already been tested in various models of post-ischemic vascularization[51–54]. Notably, in the mouse leg ischemia model, ESC-EPCs have a greater regenerative potential than undifferentiated ESCs. They are more effective after intramuscular injection than after parenteral administration[55,53]. As previously mentioned, post-ischemic revascularization requires the activation of both angiogenic and arteriogenic processes to efficiently restore tissue perfusion in the ischemic tissue. Hence, the acquisition of an endothelial specified and determined cell fate may limit the therapeutic efficiency of hESC-EPC-based therapy. In this line, combined transplantation of hESC-EPC and hESC-derived mural cells is more efficient to promote post-ischemic revascularization and maintain long-term vascular integrity than hESC-EPC alone. Therefore, the use of pluripotent stem cells still capable of dividing, while differentiating, into both endothelial and smooth muscle progenitor cells could stand as the best clinical cell source in vascular diseases. Interestingly, a purified population of multipotent cardiovascular progenitors has been recently isolated, sorted and characterized. These cardiovascular progenitors are derived from hESC treated with the cardiogenic morphogen BMP2 and Wnt3a and are depicted by the expression of OCT4, mesoderm posterior 1 (MesP1) and stage-specific embryonic antigen 1 (SSEA-1). This progenitor population was able to generate cardiomyocytes as well as endothelial cells and smooth muscle cells[56]. In this line, injection of only 10^4 hESC-derived SSEA-1+/MesP1+ cells, or their progeny obtained after treatment with VEGF-A or PDGF-BB, was effective enough to enhance postischemic revascularization in immunodeficient mice with CLI[57].

17.1.4 Induced Pluripotent Stem Cells

Recent exciting and innovative studies introduced new stem/progenitor cell source, able to differentiate into all cell types of the endo-, ecto-, or mesodermal lineages[58]. These induced pluripotent stem cells (iPSCs) can be generated from different somatic cell sources by overexpression of specific transcription factors and may open a new chapter in the field of regenerative medicine. These cells are somatic cells from adult humans that are reprogrammed to become pluripotent by transfection with genes encoding four proteins responsible for the maintenance of a state of pluripotency (oct3/4, sox2, associated with either klf4 and c-myc or with nanog and lin28)[58–60]. These transcription factors are required for, and allow, the induction of pluripotency, rather than simply its maintenance. iPSCs have been obtained from a large number of somatic cells, with subsets of these genes or other pluripotency factors, together with other small molecules that improve chromatin remodeling. Initially, iPSCs were generated by retroviral or lentiviral transduction of the reprogramming factors. The concern with this method is that incomplete silencing of the transgenes after reprogramming and possibility of integration of viral DNA into the host genome. These risks can be reduced by the use of adenoviruses, plasmid constructs, or the Cre/LoxP system. The use of non-viral methods, such as those based on reprogramming proteins, micro-RNAs, and small molecules, is also increasing. As for ESCs, human iPSCs have been differentiated into perivascular and endothelial progenitor cells (iPSC-EPCs)[61–63]. The capacity of iPSC-EPCs to improve blood perfusion in mouse models of peripheral arterial ischemic disease was rapidly demonstrated[62]. Culture in the presence of BMP4 and VEGF for 14 days, followed by the sorting of CD31-positve cells allows differentiating endothelial cells to be identified. These iPSC-EPCs can form capillaries during culture in Matrigel and incorporate acetylated-LDL. They express classical endothelial markers, such as VEGFR2/KDR, CD31, CD144, and endothelial Nitric Oxide Synthase (eNOS). The injection of iPSC-EPCs improves tissue perfusion by 30% with respect to the control group in mice with CLI. This effect is associated with a 60% increase in the total number of capillaries[62]. Transplantation of iPSCs has also been shown to restore cardiac function by promoting angiogenesis in post-infarcted swine model[64]. Finally, treatment with

iPSC-EPCs enhanced reperfusion in the ischemic limb as effectively as ESC-EPCs and bone marrow derived EPCs and more effectively than mature endothelial cells[50]. Human iPSC-ECFCs have also been found to improve blood flow recovery and limb salvage rate in nude mice[65]. Furthermore, treatment with iPSC-ECFCs was more effective in enhancing limb perfusion recovery than treatment with mature iPSC-ECs produced via embryoid body formation[65].

17.2 Canonic Mechanisms Governing Vascular Stem Cells Therapeutic Potential

17.2.1 Differentiation into Vascular Cells

The contribution of progenitor cells to new blood vessel formation is an endless subject of controversy. Genetic markers, bone marrow replacement, and parabiosis models have been used to document the existence of circulating cells that contribute to new vessel formation in the ischemic milieu[35]. With respect to negative studies, these may be related to technical issues concerning the fidelity of the engineered markers, the use of fluorescence confocal microscopy, erroneous interpretations of histomorphological images, and possible artifacts, such as the confusion between endothelial cells and inflammatory perivascular cells[66]. Furthermore, the fidelity of murine models to human physiology is well established to be variable at best. In this regard, there are data from human studies that provide supporting evidence of the role of circulating cells in vascular repair[67,68]. These data, generated by examining hearts from patients who had undergone sex-mismatched transplants, reveal that extracardiac cells may contribute to the EC population in the donor heart, with evidence that the contribution was greatest in the microcirculation and precipitated by injury signals[67,68].

Consistent with this, numerous studies reveal the capacity of cells derived from the bone marrow to differentiate into cells with an endothelial phenotype. Indeed, medullary Lin^-/c-kit^+ cells from mice expressing the green fluorescent protein integrate into cardiac ischemic tissue and differentiate into myocytes and ECs[69]. c-kit/Sca1-positive cells are incorporated into the vascular endothelium and express endothelial markers, such as CD31 and von Willebrand's factor, and thus present a functional endothelial phenotype[70,71]. Similarly, medullary mononuclear cells injected into the infarcted zone of the myocardium acquire an endothelial phenotype[72]. This capacity to differentiate may be dependent on the release of specific signals by the ischemic tissue. In particular, the generation of small membranous vesicles, such as microparticles, by apoptotic endothelial cells or endothelial cells activated by local inflammation, controls the differentiation of BM-derived cells into cells with an endothelial phenotype, probably through the production of reactive oxygen species[73].

It was initially suggested that EPCs, particularly those isolated from human peripheral blood, differentiate *in vitro* into endothelial cells and are incorporated *in vivo* into vessels at the site of active angiogenesis[8,9]. However, the relative contribution of EPCs to vascular neogenesis is highly variable (between 12 and 25% of the newly formed endothelial cells)[74–78]. Such variability and discrepancy seem to mainly depend on the existence of, at least two different types of EPCs, as described previously. In particular, early EPCs are less differentiated and do not integrate into the endothelial cell layer, instead stimulating neovascularization through their paracrine effects whereas late EPCs or ECFCs are more differentiated and are incorporated into the endothelium. They have a high proliferation potential and can form tubular structures[11,79]. Nevertheless, these two populations of EPCs probably have complementary effects because they display synergic proangiogenic effects when administered simultaneously[79].

Medullary Lin^-/c-kit^+ cells can also differentiate into smooth muscle cells and participate in vascular remodeling. BM derived cells can also differentiate, *in vitro* and *in vivo*, into smooth muscle cells[80–82]. Similarly, smooth muscle cells from donors have been detected along the atherosclerotic vessels in compatible recipients after bone marrow transplantation, providing evidence for the existence of circulating smooth muscle cell progenitors of medullary origin[83]. Finally, the intramyocardial expression of Placenta Growth

Factor by gene therapy facilitates the mobilization and recruitment of $Lin^-/Sca1^+$ cells to peri-infarct and the differentiation of these cells into smooth muscle cells[84].

17.2.2 The Paracrine Effect

However, it is likely that the major part of the beneficial effects of transplanted vascular progenitor cells for therapeutic use rely on their ability to release active entities that may influence the number, the remodeling, and the vasoactive potential of the targeted vasculature network. In this line, many experimental studies have shown that these stem/progenitor cells are not incorporated into neocapillaries, but act as support cells.

17.2.2.1 Pro-Angiogenic Factor

Adult stem cells: BM-derived MNCs injected into infarcted myocardium secrete fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and angiopoietin 1; they increase vascular and capillary density and local blood flow through tissues[72,85]. In humans, after MI, bone marrow-derived cells secrete a cocktail of more than 25 factors and cytokines with proangiogenic activities. These secreted molecules include angiogenin, VEGF-A, HGF (hepatocyte growth factor), FGF-9, and insulin growth factor-1. BM-derived cells implantation in patients with CLI increases serum levels of angiogenesis-related factors, including VEGF and nitric oxide (NO) without any correlation with the numbers of implanted cells[86]. Human and murine MSCs release a broad range of factors with proangiogenic capacities including angiogenic growth factors, proteases (MMP-1, MMP-2, MMP-9, t-PA) and factors involved in stem/progenitor cells mobilization and recruitment (Thymosine β4, stem cell factor, G-CSF)[87–90].

Conditioned medium from an early EPC culture has proangiogenic effects in ischemic legs resembling those observed after EPC injection[91]. The paracrine activity of EPCs includes, above all, the release of growth factors likely to initiate vascular neogenesis in the ischemic territory. EPCs secrete many molecules, including VEGF, HGF, G-CSF and GM-CSF into the extracellular medium[92]; they also release proteases, such as cathepsin L, metalloproteinases, and the urokinase plasminogen activator (uPA) responsible for degradation of the extracellular matrix and release of the growth factors sequestered in the extracellular matrix[93–95]. Inhibition of the activin receptor-like kinase 1 reduces EPCs proangiogenic effect whereas EPCs exposed to either short- or long-term stimulation with its high-affinity ligand, the bone morphogenetic protein 9, markedly improve revascularization in mice with CLI[96]. Mice lacking cathepsin L display a modification of tissue revascularization processes following leg ischemia. In addition, EPCs from mice with an invalidation of the cathepsin L gene do not integrate into the site of ischemia and do not increase post-ischemic revascularization[97]. EPCs express proteinase-activated receptors (PARs) 1 and 2, which act as thrombin receptors, and their activation favors all the steps of angiogenesis[14,98]. Namely, the activation of PAR-1 on EPCs increases angiogenesis *in vitro*, by activating angiopoietin 2 pathways, thereby favoring cell proliferation, and the CXCL12/CXCR4 pathway, which promotes cell differentiation and migration[99]. The mechanism of action of SMPCs, although less well described and analyzed than that of EPCs, is likewise based on the paracrine release of vasoactive molecules. Hence, injection of SMPCs derived from mononuclear cord blood cells does not result in the recruitment of these cells to the hypoxic zone or the activation of a post-ischemic revascularization process[48]. A proteomic analysis of the secretome of these cells revealed that they produced few proteolytic enzymes and inflammatory cytokines and that they therefore had only a limited invasive capacity. The conditioned medium obtained after the culture of SMPCs also had an extremely limited angiogenic potential for the formation of endothelial tubes[100]. By contrast, the administration of SMPCs synergizes the effect of EPCs injection in a mouse model of leg ischemia[48]. SMPCs seem to release angiopoietin-1, which binds to its receptor, Tie-2, on the surface of EPCs, promoting the recruitment and incorporation of EPCs into the pre-existing vascular network[48].

17.2.2.1.1 ESCs Several studies have demonstrated an activation of post-ischemic revascularization by ESC-ECs through paracrine effects[53,55]. Genome-wide Affymetrix GeneChip analysis revealed that the ESC-EPCs express a wealth of secreted factors known to induce angiogenesis, tissue remodeling, and organogenesis[54]. Encapsulation of hESC-EPCs in Matrigel blocks cell migration and extravasation but treatment with encapsulated hESC-EPCs is sufficient to suppress limb loss and tissue damage, confirming a major role of the paracrine activity[53].

17.2.2.1.2 iPSCs Canine iPSC-EPCs increase blood perfusion recovery in mice with CLI at 7 and 14 days after delivery, although transplanted cells almost disappeared by day 14[101]. Similarly, the number of intramuscular injected human iPSC-EPCs was greatly reduced by day 7 and undetectable by day 11, which prompted the inclusion of an additional cell treatment at day 7[62]. Hence, as for other type of stem/progenitor cells, iPSC-EPCs most likely exert their pro-angiogenic effects primarily through paracrine mechanisms and to a lesser extent through engraftment into existing vasculature. Consistent with this, hiPSC-EPCs secrete major angiogenic factors including epidermal growth factor, hepatocyte growth factor, vascular endothelial growth factor, placental growth factor, and CXCL12. Of interest, the magnitude of cytokine upregulation upon hypoxic is more dramatic in hiPSC-EPCs compared to BM-derived EPCs underlying their major therapeutic potential[101].

17.2.2.2 Vasoactive Factors

Cells of medullary origin have similar effects on smooth muscle cells, activating remodeling of the existing vascular network[89]. These cells also release vasoactive molecules, such as NO. NO induces the vasodilation of pre-existing vessels and increases vascular permeability, thereby improving the extravasation of the circulating blood cells, stem, and progenitor cells participating in post-ischemic vascular remodeling and revascularization[102]. BM derived cells and CD34+-derived progenitor cells interacted with ischemic femoral arteries through the chemokine CXCL12 and its receptor CXCR4 and released NO via an eNOS-dependent pathway. BM derived cells-induced NO production promotes a marked vasodilation and disrupts vascular endothelial-cadherin/beta-catenin complexes, leading to increased vascular permeability. NO-dependent vasodilation and hyperpermeability are critical for BM-derived cells infiltration in ischemic tissues and their proangiogenic potential in a model of hindlimb ischemia in mice[102]. There are also pharmacological compounds that activate transcription of the eNOS gene, such as AVE9488, which may induce the differentiation of stem cells into hematopoietic and endothelial cells; such agents could be used in the pretreatment of cells from ischemic patients[103]. An increase in eNOS levels in EPCs improves their regenerative potential in ischemic muscle[102,103]. The overproduction of eNOS increases the proangiogenic potential of mononuclear cells of medullary origin and is sufficient to restore their activity in a context of diabetes and hypercholesterolemia[104]. Tetrahydrobiopterin (BH_4) is an essential cofactor of eNOS. BH_4 reduction results in eNOS uncoupling and the generation of superoxide instead of NO. Intracellular BH_4 levels are regulated by the *de novo* biosynthetic pathway from guanosine triphosphate and GTP cyclohydrolase I (GTPCH I) is the rate-limiting enzyme. Overexpression GTPCH increases migration, tube formation and pro-angiogenic potential of BM- or blood- derived EPCs[105]. GTPCH/BH4 pathway critically regulates EPC number and function, at least in part, via suppressing thrombospondin-1 expression and oxidative stress[106].

17.2.2.3 Extracellular Membrane Vesicles

There is mounting evidence to suggest that stem cells can also release extracellular membrane vesicles (EVs) that may contain both autocrine and paracrine angiogenic factors. Two types of EVs should be considered: exosomes are some of the smallest of these EVs and are often described as having a size of the order of

30–100 nm, while microparticles (MPs) are generally between 100 and 1 μm. These size ranges, however, are not considered absolute. The mechanism of release of these different particles is also different, in that exosomes are produced through the endosomal pathway, whereas MPs are released through budding from the cell membrane. Hence, BM cell extract prepared by subjecting BM-derived cells to three freeze-thaw cycles followed by microcentrifugation enhance vessel growth to a similar extend than that of intact BMC therapy[107]. Similarly, murine and human irradiated ESCs, despite irradiation doses that prevented proliferation and induced cell death, significantly improved cardiac function and decreased infarct size compared with untreated or media-treated controls[108]. *In vitro*, exosomes collected from the conditioned media of mobilized human CD34+ cells mimic the angiogenic activity of CD34+ cells, by increasing endothelial cells survival, proliferation, and tube formation[109]. Inhibition of EVs release impaired CD34-positive PBMNCs proangiogenic effects[110]. Adipose MSC-derived EVs, as well as bone marrow MSC-derived EVs, promote angiogenesis in ischemic tissue[111,112]. Hypoxic stimulation of the EVs-producing stem cells was required to obtain functional EV[112]. Similar effects of hypoxia were observed in EVs from human UCB-MSC, which promote angiogenesis *in vitro* as well as *in vivo* in rat with CLI[113]. These findings underline the importance of tissue microenvironment that may fine-tune the ability of transplanted stem cells to release EVs as well the nature and the content of these locally secreted EVs.

These vesicles may contain key determinant necessary to maintain stem cell properties and their quantitative reduction or loss may result in cellular differentiation or phenotypic changes[114]. Alternatively, extracellular membrane vesicles carry on their surface some of the cell surface markers of their cell of origin and evidence is mounting that they are able to interact with the cell surface receptors on neighboring and possibly also distant cells. In addition, they are able to carry a cargo, which includes proteins, messenger RNAs (mRNAs), and microRNAs (miRNAs) and to transfer these cargos to recipient cells, thus contributing to cell-to-cell communication. Hence, these vesicles may transfer specific information through for example, targeted microRNAs delivery using MPs, to cells located in the ischemic environment[115]. In this line, saphenous vein-derived pericyte progenitor cells-secreted miRNA-132 triggers the angiogenic program in cardiac tissue after myocardial infarction[116]. EPC-derived EVs increase endothelial cell proliferation, migration, and vessel formation *in vitro* by transfer of pro-angiogenic miRNAs, miR-126, and miR-296. These EPC-derived EVs enhance capillary density, enhanced limb perfusion, and reduced injury after 7 days in mice with CLI[117]. However, the role of such miRNAs is also likely dependent of the type of stem-progenitor cells. Hence, although the miRNA-17–92 cluster regulates vascular integrity and angiogenesis, none of this member has a significant impact on the endothelial differentiation of murine embryonic stem cells or induced pluripotent stem cells[118].

17.2.3 Interaction with the Host Tissue

Interactions between stem/progenitor cells and the host tissue may contribute to the overall effect of these cells on vascular neogenesis. Treatment with cardiac-derived stem cells has been shown to reduce the number of CD68-positive macrophages, and to secrete factors that polarized macrophages toward a distinctive cardioprotective phenotype[119]. Hence, transplanted vascular stem cells from different origin may also coordinate inflammatory cells number and activation mode, which play an instrumental role in the control of vessel growth in the ischemic tissue[7,120–123]. Numerous cytokines have been identified among the many factors secreted by cells of medullary origin in the ischemic territory including cytokines (IL-1, TNF − α IL-10) and chemokines (CCL-2, CCL-23, CCL-24, CXCL-6, CXCL-12, and CXCL-13)[72,88,124]. BM-derived MSCs improve vascular regeneration through an alteration in the number of rolling and sticking leukocytes in a critically ischemic murine skin flap model[125]. Such an effect could also be mediated by EVs since EVs-derived human MSCs are internalized by circulating mononuclear cells, and significantly decrease the levels of pro-inflammatory cytokine IFN-γ but increase that of FoxP3-positive regulatory T-cells[126]. Interestingly, regulatory T-cells have been shown to modulate post-ischemic vessel growth[127].

In mice treated with human UCB-EPCs, gene analysis with probes specific for human or mouse genes has revealed that human UCB-EPCs have persistent paracrine activity during the first 7 days following their injection into ischemic tissues in mice. The host tissue then takes over and host cells essentially produce growth factors in the ischemic zone[55]. The paracrine factors released by human UCB-EPCs can also induce the mobilization and endogenous recruitment of stem/progenitor cells of medullary origin[55]. In this line, exogenous administration of PB-MNCs raises the number of endogenous BM-derived circulating vascular progenitor cells as well as their pro-angiogenic potential in diabetic nude mice with CLI[128].

The injected EPCs may also activate the migration and proliferation of stem cells resident in the ischemic tissue. For example, human EPCs injected intravenously are recruited to ischemic leg muscle, where they secrete VEGF, CXCL12, and IGF-1, promoting the migration of c-kit-positive resident cardiac progenitor cells[129]. The administration of BM-derived c-kit-positive cells stimulates the emergence of new cardiomyocyte from endogenous cardiogenic progenitor activity[130].

Finally, the injected cells may interact with the environment at some distance from the ischemic zone. After intravenous injection of MSCs, most of the cells are trapped in the lungs and disappear within about 24 hours. Human MSCs induce the expression of many genes in the pulmonary tissue, including that encoding the anti-inflammatory protein TSG-6. Human MSCs treated with siRNAs directed against TSG-6 have no positive effect on cardiac remodeling and treatment with a recombinant TSG-6 protein decreases the size of the infarct and the inflammatory response. Thus, the pulmonary activation of MSCs and the resulting production of TSG-6 contribute to the beneficial effects of these cells on post-ischemic tissue regeneration[131].

17.3 Stem-Cell-Based Therapies in Patients with Peripheral Artery Disease

These exciting preclinical development of stem cell-based approaches for therapeutic revascularization led to rapid initiation of clinical trials in humans, as early as 2002[132]. The stem/progenitors cells were obtained from an autologous preparation of mononuclear cells from the bone marrow, and the pathological target was critical ischemia of the legs. In this first published clinical trial[132], a 500 ml bone marrow sample was removed under general anesthesia, and the mononuclear cells were then isolated and concentrated to a final volume of 30 ml. The total number of cells injected was between 0.7 and 2.8×10^9. During this pilot study on 25 patients with CLI, cells were administered within 3 h of sampling, via 40 local intramuscular injections into the ischemic leg. Given the very positive results obtained in this open study, a second group of patients was included, for a randomized study. This group consisted of 22 patients with bilateral leg ischemia. The 44 legs of these 22 patients were randomized and treated with mononuclear cells from the bone marrow or mononuclear cells from the peripheral blood. The patients were followed for 4 weeks after the transplantation procedure and then at 4-month intervals. After 4 and 24 weeks, a significant improvement of all the clinical criteria and the formation of collateral vessels, as demonstrated by angiography, were observed in all the legs treated with medullary cells, but not in the control legs treated with peripheral blood mononuclear cells. The ischemic ulcers and gangrene regressed markedly. This study showed, for the first time, that the implantation of bone marrow mononuclear cells is both effective and safe in patients with leg arteritis[132].

Many studies using the same protocol have since confirmed the feasibility and safety of cell therapy with mononuclear cells and/or marker selected cells isolated from the bone marrow or the peripheral blood (for a review, see ref.[7]). In two feasibility studies, the activation of angiogenesis following the injection of bone marrow mononuclear cells was demonstrated, by analyses of amputated material from the treated patients. These foci of active angiogenesis were detected at sites of injection or at the ischemic extremities[133–135]. Moreover, presence of these foci of active angiogenesis have been correlated to bone marrow EPC content[136]. After more than a decade of cell therapy in PAD, genuine clinical efficacy has yet

to be demonstrated in large-scale, randomized, placebo-controlled studies of a sufficiently large size for evaluation of the real impact of the treatment on morbidity and mortality. In this chapter, we will focus on randomized studies reported in PAD (Table 17.1).

Table 17.1 *Randomized studies reported in peripheral arterial disease*

Comparisons	Delivery method	Patient number	Follow-up	Outcome/results	Refs
BM-MNC vs Placebo					
Tateishi-Yuyama et al. (TACT)	IM	25	24 weeks	improvement in ABI and pain	132
Walter et al. (PROVASA)	IA	40	6 months	improvement in ulcer healing and pain	140
Teraa et al. (JUVENTAS)	IA	160	6 months	negative study	141
Iafrati et al.	IM	48	3 months	improvement in amputation, pain, ABI	142
Benoit et al.	IM	48	6 months	lower amputation rates	143
Prochazka et al.	IM	96	4 months	improvement in amputation and ABI	144
BM-MNC: IA vs IM					
Van Tongeren et al.	IA + IM vs IM	27	12 months	improvement in ABI and pain in both groups	139
BM-MNC vs other cell type					
Tateishi-Yuyama et al. (TACT) *vs PB-MNC*	IM	22	24 weeks	improvement in ABI and pain	132
Matoba et al. (TACT) *vs PB-MNC*	IM	115	3 years	improvement in ulcer healing and pain	148
Huang et al. *vs PB-MNC*	IM	450	3 months	improvement in ulcer healing and pain in PB-MNC	149
Kamata et al. *vs PB-MNC*	IM	6	1 month	improvement in pain	150
Lu et al. *vs BM-MSC*	IM	41	6 months	improvement in ABI and pain for both cell type - faster ulcer healing with BM-MSC	155
Perin et al. *Vs ALDH*br *cells (CLI-001)*	IM	21	6 months	no difference between cell therapy: Improvements of ABI in both groups	154
Kirana et al. *Vs ixmyelocel-T*	IM/IA	24	12 months	no difference between cell therapy improvements of microcirculation and wound healing in both groups	158
PM-MNC vs Placebo					
Huang et al.	IM	28	3 months	improvement in ulcer healing, pain and amputation rates	145
Ozturk et al.	IM	40	3 months	improvement in ABI and pain	146
Mohammadzadeh et al.	IM	21	3 months	improvement in ABI and amputation rates	147
Other cell type vs placebo					
Losordo et al. (ATCD34-CLI) *CD34+*	IM	28	12 months	no improvement in amputation rates with increased cell doses	152
Powell et al. (RESTORE-CLI) *ixmyelocel-T*	IM	86	12 months	decreased amputation rates, mortality and gangrene	157

17.3.1 Mononuclear Cells from Bone Marrow and Peripheral Blood

One of the first questions about cell therapy in PAD is the route of delivery. Most clinical studies have used an intramuscular (IM) mode of MNC delivery in patients with PAD[7]. Intramuscular delivery is hypothesized to result in a transient cell engraftment directly within the ischemic tissue site to allow both local paracrine activity as well as incorporation of cells into the neovasculature. Intra-arterial (IA) administration has also been tested. IA delivery involves a stem cells homing into ischemic and peri-ischemic zones. Intra-arterial delivered BM-MNC to ischemic limbs has been shown to result in increased capillary densities and improved ankle brachial pressure index and pain-free walking distance at 1 year[137,138]. IM and combined IM/IA delivery of autologous BMC have been compared in a randomized study and both sustained improvement in a considerable proportion of patients with severe PAD who are not amenable for conventional treatment[139]. This feasibility and efficacy of intra-arterial injections of bone marrow mononuclear cells has been confirmed in PROVASA study. Indeed, Walter et al. injected progenitor cells transplantation of bone marrow mononuclear cells for induction of neovascularization in patients with peripheral artery occlusive disease study with intra-arterial injection[140]. This randomized, placebo-controlled study also assessed the efficacy of the repeated injection of cells, comparing the results obtained with single injection. The treated patients displayed no significant improvement in systolic blood pressure index, but did display considerably enhanced ulcer healing and less pain than the "placebo" group. Clinical improvement is also correlated with the levels of CD34+ cells as well as the early EPC colony formation in the bone marrow. This finding is consistent with the observation of an increase in the number of early EPCs obtained in culture for patients treated with bone marrow mononuclear cells and presenting high levels of revascularization[136].

Randomized trials of BM-MNCs[132,140–144] or PB-MNCs[145–147] versus placebo have confirmed early clinical improvements (3 months) in ankle brachial pressure index and pain, although amputation rates improvement differ between studies. Concerning PB-MNC, only one study reported significantly fewer patients facing major amputation of a limb in the group infused with PB-MNC versus those in the control group at the conclusion of the studied[147]. The larger randomized study in PAD has been recently published[141]. The JUVENTAS (Intra-arterial Stem Cell Therapy for Patients with Chronic Limb Ischemia) study included 160 CLI patients treated with intra-arterially administered BM-MNC with a follow-up of 6 months. Repetitive intra-arterial infusion of autologous BMMNCs into the common femoral artery did not reduce major amputation rates in patients with severe, non-revascularizable limb ischemia in comparison with placebo. The safety outcome (all-cause mortality, occurrence of malignancy, or hospitalization due to infection) was not significantly different between the groups. Secondary outcomes quality of life, rest pain, ankle-brachial index, and transcutaneous oxygen pressure improved during follow-up, but there were no significant differences between BM-MNC and placebo. This study makes the relevance of cell therapy approach in CLI with BM-MNC hard to believe for the future.

Concerning cell population comparison, four randomized controlled trials have directly compared BM-MNC with PB-MNC[132,148–150]. As previously described, the first study by Tateishi-Yuyama et al.[132] used CLI patients with bilateral ischemia as their own controls to directly compare the therapeutic effects of BM-MNC and PB-MNC in CLI patients out to 1 year. They showed that BM-MNC infusion resulted in significant improvements in ankle brachial pressure index, rest pain, pain-free walking time compared with both the placebo group, and with those infused with PB-MNC. Huang et al reported that at 3 months[149], patients treated with PB-MNC showed improved ankle brachial pressure index and rest pain compared with patients receiving BM-MNC. Matoba et al, reporting on long-term follow-up of the TACT study, revealed significant improvements in pain, walking distance, and ulcer size at 2 years in patients infused with BM-MNC compared with those treated with PB-MNC[148].

17.3.2 Selected Cell Population

Numerous pilot and/or phase I/II studies have also been carried out to evaluate the efficacy of bone marrow CD133+ cells, peripheral blood mononuclear cells, mobilized CD34+, bone marrow-derived aldehyde dehydrogenase bright cells, or MSCs for patients with CLI or Buerger's disease (TAO, thromboangiitis obliterans). Clinical safety and feasibility of CD34+ cells for ischemic limb treatment have been evaluated in a dose escalation trial of granulocyte-colony stimulating factor–mobilized peripheral blood CD34+ cells (3 doses: 10^5, 5×10^5, 10^6) administered by intramuscular injection[151]. Any dose of CD34+ administered resulted in a total efficacy score improvement at 3 months, exhibited by pain and ulcer size reduction, and increases in toe brachial pressure index, and pain-free walking distance. Longer term clinical benefits were then tested in a 28-patient, randomized, double-blind, controlled, dose-escalation study in which granulocyte-colony stimulating factor-mobilized selected CD34+ cells were administered by intramuscular injection (ACT-34 CLI [Autologous Cell Therapy-34 Critical Limb Ischemia] trial)[152]. The study showed that at 12 months, amputation incidence was lower in the combined cell-treated groups (doses of 10^5 or 10^6 cells per kg body weight) compared with the control group ($P=0.054$). Additionally, each dose group trended toward improved amputation-free survival at 6 and 12 months.

Another heterogeneous mix of MNC derived proangiogenic cell populations has been explored using high aldehyde dehydrogenase (ALDH) activity. ALDH is an oxidizing enzyme highly expressed in both embryonic and adult stem cells, and BM-MNCs selected for high ALDH activity express CD34+, CD133+, CD14+, and CD117+[153]. A phase 1, randomized, controlled trial compared unselected BM-MNCs to BM-MNCs selected for high ALDH in the treatment of CLI, demonstrated significant ankle brachial pressure index improvement in both groups[154].

The therapeutic potential of MSC transplantation has also been explored in clinical trials. A randomized controlled trial comparing BM-MSCs to BM-MNCs was conducted on 41 CLI patients with diabetes mellitus and demonstrated significantly improved pain-free walking time in both groups at 6 months[155]. BM-MSC infusion led to significantly greater collateral blood vessel scores and a faster ulcer healing than patients receiving BM-MNC. In addition to allogeneic MSCs, various cell types such as adipose-derived regenerative cells (ADSC), induced pluripotent stem cells, and nuclear reprogrammed stem cells are currently being explored for proangiogenic potential for PAD treatment in preclinical models. Phase 1 studies of autologous ADSC transplantation in patients with objectively proven CLI not suitable for revascularization have demonstrated their feasibility and safety[156]. Randomized trials with these ADSC are under investigations, but results and outcomes of these studies have yet to be reported.

A mix of multiple cell lineages for PAD treatment has also been studied. Ixmyelocel-T has been tested in randomized studies in PAD. Ixmyelocel-T is an expanded autologous multicellular therapy, manufactured from a small sample of bone marrow aspirate. Ixmyelocel-T contains expanded populations of MSCs and M2-like macrophages, as well as many of the CD45+ cells found in the bone marrow. In a randomized phase 2 trial (RESTORE-CLI), this mixed cell preparation Ixmyelocel-T was administered intramuscularly to CLI patients in comparison to placebo. These cells resulted in a significantly prolonged time to treatment failure and trend toward increased amputation-free survival at 1 year[157]. In a small randomized trial, Kirana et al. explored Ixmyelocel-T-cell versus BM-MNC in CLI patients but no differences between the two groups were observed[158].

17.3.3 Endothelial Progenitor Cells

EPCs have been the first type cells proposed for autologous angiogenic cell therapy and have demonstrated their ability to migrate to ischemic tissue and contribute to neovascularization in response to tissue ischemia[8,159]. However, the cell type responsible for the observed proangiogenic effect of total MNC or their subpopulation

(CD133+ or CD34+) and the subsequent clinical benefit remains unclear[160]. To date, clinical data on MNC from BM or PB never allow us to determine the real active cellular component. EPCs circulate in blood as rare events, and cell characterization requires a culturing step that enables the identification of at least two populations of EPCs[11]. "Early" EPCs appear within 4–7 days of culture, are spindle-shaped, and express both endothelial and leukocyte markers. This cell population can be quantified in culture as described by Hill[161] and has been reported to grow around a core of T lymphocytes[162]. The number of these so-called "colony-forming unit-endothelial cells" in peripheral blood has been found to negatively correlate with cardiovascular risk factors[161,163]. Despite their poor relevance in vasculogenesis and their absence, now well admitted, of real ability to differentiate into vascular endothelial cells, these cells have been tested in clinical cell therapy trials for cardiac diseases[164–171]. These early EPC or angiogenic cells have never been tested in PAD. Endothelial colony-forming cells (ECFCs), also known as "late" EPCs, have been studied to a far lesser extent than "early" EPCs[172]. They develop after 2–3 weeks of culture and share characteristics of precursors committed to an endothelial lineage. ECFCs have been isolated from the blood of patients with CAD[173], acute myocardial infarction[174], and pulmonary hypertension[175] but their therapeutic potential has never been tested in clinical trials because of inherent difficulties to isolate and expand them in sufficient number allowing their use through clinical approved procedures[176].

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18

Gene Modified Stem/Progenitor-Cell Therapy for Ischemic Stroke

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18.1 Introduction

Stroke is one of the leading causes of death and disability. There are two types of stroke, ischemic and hemorrhagic. Ischemic stroke is the major type in clinical patients, with a incidence rate of more than 70% of all stroke cases. Multiple factors can lead to stroke [1, 2]. These risk factors include aging, smoking, hypertension, diabetes, hypercholesterolemia, and lack of exercise [3]. The brain is very sensitive to ischemia-induced oxygen and glucose deprivation. Neurological functions are impaired within minutes after stroke onset, and these deficits can last from hours to years. After stroke onset, many pathophysiological events happen, which include, but are not limited to, failure of energy supply, the degradation of the blood-brain barrier (BBB), disruption of cell ion homeostasis, release of excitatory amino acids, increase in intracellular calcium levels, activation of gill cells, and infiltration of inflammatory cells [4, 5]. These interrelated events collectively lead to non-selective brain cell necrosis and apoptosis, which results in the loss of neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells [6].

The volume and location of brain ischemia determine the long-term functional deficits of patients [7]. The penumbra is the region regarded as having the potential to be rescued by reperfusion or post-acute therapies [8]. Until now, the only FDA approved non-invasive treatment for ischemic stroke has been the intravenous delivery of recombinant tissue plasminogen activator (rtPA). However, the short treatment window (less than 4.5 h) limits the number of patients that could benefit from it. Several on-going trials are testing the safety and efficacy of stem cell treatments and gene therapy treatment, which might extend the treatment window for ischemic stroke.

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In this chapter, we discuss the pre-clinical research and clinical trials of several kinds of gene modified adult stem/progenitor cell therapy for ischemic stroke. The gene modification methods discussed here include viral and non-viral vectors and limitations to gene modified stem/progenitor cell for ischemic stroke.

18.2 Gene Modified Stem Cells for Ischemic Stroke

The term "stem cell" was first used in 1908 by Alexander Maksimov, stating that there was a putative existence of self-renewing precursor cells in bone marrow [9]. Stem cells are broadly classified into three classes: embryonic stem cells derived from the inner cell mass of blast cysts, adult stem cells in various tissues, and induced pluripotent stem cells produced from adult somatic cells by transduction of embryogenesis-related genes [10, 11]. Tissue specific adult stem/progenitor cells have been discovered in various tissues; these include hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, and neural progenitor cells [12]. Under given physiological or experimental conditions, these cells have the potential to differentiate into tissue- or organ-specific cells with special properties. Stem cells can not only contribute to tissue recovery as basic building blocks but also can be used as carriers for gene therapy. Recent years have seen the rise of stem cell based gene therapy as a new strategy that can capitalize on the advantanges of both. Many types of stem cells have been used as carriers for gene therapy.

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the developing blastocyst embryos and give rise to all derivatives of the three primary germ layers, which can differentiate into nearly all cell types during development [13, 14]. ESCs can be indefinitely expanded *in vitro*. They can be induced to neural stem/progenitor cells. Given the specific differentiation potentials, transplantation of ESCs has been used to treat spinal cord injury in a rat model, where they were shown to differentiate into neurons, astrocytes, and oligodendrocytes, while acchieving partial functional recovery [15]. Owing to their unlimited differentiation potential, ESCs could provide abundant supplies of a wide variety of cell types for stem cell based gene therapy. However, ESC research has been hindered due to safety and ethical concerns. The safety concerns are due to the potential of ESCs to form teratomas and tumors when undifferentiated or when partially differentiated ESCs were transplanted *in vivo* [16, 17]. Immune response could also occur when allogeneic ESCs were transplanted *in vivo*, which may require immunosuppression in the recepient [16]. The ethical concerns related to ESCs are mainly due to the fact that early sources of ESCs were from human embryos. Following the development of embryo, the potency of the ESCs begins to narrow down to "fate determined stem cells," which give rise to cells of a particular tissue. For example, neural stem cells differentiate into the nervous lineage cells and hematopoietic stem cells into blood cells [18]. These fates determined stem/progenitor cells retain a limited capacity to proliferate and differentiate, with reduced risk of tumorgenesis. In past decades, a variety of adult stem/progenitor cells has been defined both in adult animals and humans, such as mesenchymal stem cells, neural stem/progenitor cells, endothelial progenitor cells, and oligodendrocyte progenitors [19–21]. The adult stem/progenitor cells have some advantages over ESCs. First, adult stem/progenitor cells can be transplanted autologously without eliciting significant immunologic response. Second, the adult stem cells have a predetermined cell fate. Under controlled conditions they can only differentiate into specific cells, reducing the risk of tumorgenesis. Third, ethical issues regarding the source of adult stem/progenitor cells are not controversial like the case of ESCs is [22]. Collectively, adults stem/progenitor cells are becoming the new candidates for the development of stem cell based gene therapy treatment.

The concept of stem cell based gene therapy was first proposed in 1997 [23]. And after that, stem-cell treatment has brought us a historic avenue in gene therapy for various diseases by using different stem/progenitor cells modified by a variety of genes, depending on the disease. Most gene transfer strategies to stem/progenitor cells will be performed *in vitro*, then delivery of the genetically modified stem/progenitor

cells *in vivo*. The combination of gene therapy and stem cell therapy by modification of stem cells with specific genes may have additional efficacy to stem cell or gene therapy alone. The modified cells are able to differentiate and may act as sources for the transgene product. *Ex vivo* gene transfer to stem/progenitor cells provides some unique advantages over the direct gene transfer *in vivo*. *Ex vivo* transfer allows researchers to work only with cells to sort the successful transferred cells and to make a quality control of the therapeutic agents. On the other hand, researchers can engineer a controllable cell line where some genes can be turned on or turned off according to requirements [24]. Mesenchymal stem cells, endothelial progenitor cells, neural stem/progenitor cells, and even induced pluripotent stem cells are known to be beneficial for ischemic stroke and have been studied as gene modification candidates.

18.2.1 Gene Modified Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) were first reported in the 1960s, and were named osteogenic stem cells or bone marrow stromal stem cells. These were discovered as a subgroup of bone marrow cells. MSCs adhered to the culture plate, presented a fibroblast like appearance, and formed colonies *in vitro* [25, 26]. MSCs represent a very small portion of the total bone marrow mononuclear stem cell population, ranging from 0.001 to 0.01% of the total cell population. This means approximately 2–5 MSCs per 1×10^6 mononuclear cells [27, 28]. Many years after MSCs were first discovered, the term MSCs was first used by Caplan in 1991 [29]. Since then, MSCs have been found to enrich several different tissue types including skin, fat, muscle, dental, umbilical cord, and placenta, among others [30–32]. Owing to the heterogeneity of MSCs, it has been difficult to establish a precise definition. MSCs lack a unique cell surface marker. Some criteria should be followed in order to classify MSCs. First, MSCs must be plate-adherent when cultured under standard conditions. Second, they must be CD105, CD73, and CD90 cell surface marker positive cells, but CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR cell surface marker negative cells [33]. Moreover, they present the ability to differentiate into mesodermal lineages cells, including osteoblasts, adipocytes, and chondroblasts *in vitro* [33]. Besides that, MSCs are believed to have a regenerative capacity in myocardial ischemia and also have immune-suppressive effects [34]. MSCs may function by paracrine mechanism or by differentiation and regeneration by themselves. The exact mechanisms have not been fully elucidated yet.

MSCs are very easily harvested and cultured *in vitro*. They are able to modulate the host immune system by lymphocyte regulation and the suppression of inflammatory cytokine release from cells of the innate immune system [35]. MSCs are characterized as quiescent and non-cycling with low cell turnover. Due to these characteristics, MSCs have been explored as a promising target for tissue regeneration stem cells [36]. For example, to deliver specific secreted protein to a local site and to provide support for other tissues. Genetically modified MSCs have been shown to serve as an effective vehicle for the replacement of genes responsible for regeneration aims [37]. Ischemic stroke leads to neurotrophic factor deprivation, and blood vessel cell and neuronal death. Many neurotrophic factors and pro-angiogenesis and pro-neurogenesis factors have been used to modify MSCs for ischemic stroke treatment in pre-clinical research (Table 18.1).

The brain derived neurotrophic factor (BDNF) gene modified MSCs by using a fiber-mutant F/RGD adenovirus vector and it was found that the BDNF protein was 23-fold greater than that of untransfected MSCs. The rats used underwent middle cerebral artery occlusion (MCAO) and received MSC-BDNF: they showed greater functional recovery than the control group. Meanwhile, MRI analysis data suggested that MSC-BDNF treated ischemic rats showed significant recovery after 7 and 14 days. Furthermore, the number of TUNEL-positive cells in the ischemic peril-infarct was also smaller in the MSC-BDNF treated rats than the control group [38]. Glial cell line-derived neurotrophic factor (GDNF) modified MSCs was grated into the ischemic rats after 3 days of ischemia, which significantly improved behavioral function compared

Table 18.1 *Gene modified stem cells therapeutic in human diseases or animal models*

Model	Stem cell type	Gene modification	Gene deliver vector	Transplantation time point and route	Outcomes	Ref
Rat tMCAO (90 min)	Human MSCs	BDNF	adenovirus	24 h after IS; intracerebral	↑functional recovery; ↓TUNEL cells.	[38]
Rat tMCAO (2 h)	Rat MSCs	GDNF	plasmid	3 d after IS; caudal veins	↑functional recovery; ↓apoptosis.	[39]
Rat tMCAO (2 h)	Rat MSCs	HGF	multimutated herpes simplex virus type-1 vector	2 h or 24 h after IS; Intracerebral	↓neurological deficits; ↓infarct volume; ↓apoptosis cells; ↑remaining neurons.	[40]
Rat pMCAO	Human MSCs	PIGF	fiber-mutant F/RGD adenovirus vector	3 h after IS; intravenous	↓lesion volume; ↑angiogenesis; ↑functional improvement.	[41]
Rat tMCAO (2 h)	Rat MSCs	VEGF	replication-deficient herpes simplex virus type 1	24 h after IS; intracerebral	↑functional recovery; ↓infarct volume; without aggregating edema.	[42]
Rat pMCAO	Human MSCs	Ang-1	adenovirus	6 h after IS; intravenous	↓brain lesion volume; ↑Functional recovery; ↑angiogenesis, neurovascularization, and regional blood flow.	[43]
Rat pMCAO	Human MSCs	Ang-1 and VEGF	fiber-mutant adenovirus vector	6 h after IS; Intravenous	↑structural-functional recovery.	[44]
Rat MCAO	Rat MSCs	CXCR4	lentivirus	24 h after IS; femoral vein	↑MSCs in ischemic boundary; ↑capillary density; ↓infarct volume; ↑neurological function.	[45]
ICH mice	NSC Cell line	VEGF	retrovirus	— intracerebral	↑functional recovery; ↑renewed angiogenesis.	[61]
Rat tMCAO (2 h)	Rat NSC	NT-3	lentvirus	7 d after IS; intracerebral	↑functional recovery.	[63]
Rat tMCAO (2 h)	Rat NSC	GDNF	plasmid	3 d after IS; intraventricular	↑functional recovery; ↓Lesion volume; ↑Syp and PSD-95 immunoreactive product, BDNF and NT-3 protein; ↓caspase-3 and TUNEL positive cells.	[64]
Rat tMCAO (2 h)	Rat NSCs	BDNF	retrovirus	3 d after IS; intracerebral	↑Functional recovery; differentiated into mature neurons after 12 weeks of transplantation.	[65]

(*Continued*)

Table 18.1 *(Continued)*

Model	Stem cell type	Gene modification	Gene deliver vector	Transplantation time point and route	Outcomes	Ref
diabetic mice pMCAO	Mice EPC	CXCR4	adenovirus	2 h after IS; Tail vien	↑the efficacy of EPC infusion; ↓infarct volume; ↑angiogenesis and neurogenesis.	[89]

Ang-1 = angiopointin-1; BDNF = brain derived neurotrophic factor; d = day; EPC = endothelial progenitor cell; GDNF = glial cell line-derived neurotrophic factor; h = hour; HGF = hepatocyte growth factor; ICH = intracerebral hemorrhage; IS = ischemia; min = minute; MSC = mesenchymal stem cell; NSC = neural stem cell; NT-3 = neurotrophin-3; PIGF = placenta growth factor; pMCAO = permanent middle cerebral artery occlusion; tMCAO = temporary middle cerebral artery occlusion; VEGF = vascular endothelial growth factor; ↑ = increase; ↓ = decrease; — = not mentioned.

to the controls 3 days after transplantation. After 14 days of transplantation, the rat brains that received MSCs-GDNF showed more effectivity in reducing apoptotic cells and enhancing Bcl-2 expression, which were recognized beneficial for brain recovery [39]. Transplantation of hepatocyte growth factor (HGF) gene transferred MSCs into ischemic rat brains after MCAO, showed significant improvement in neurological deficits and decreased infarct volume, compared with the phosphate-buffered saline treated group and MSC only treated group 14 days after transplantation [40]. Another neuroprotection factor, placenta growth factor (PIGF), was also identified to boost the therapeutic effect of MSCs by their modification. MSC-PIGF transplantation in cerebral ischemic rats showed that PIGF-MSC reduced lesion volume, induced angiogenesis, and elicited functional improvement compared with non-modified MSCs [41]. Two hours after transplantation, abundant expression of hepatocyte growth factor (HGF) protein in the ischemic brain of the MSC-HGF group was found compared with others on day 1 after treatment and was maintained for at least 2 weeks [40]. So we need to take the treatment time point carefully to gain the best protein expression time course.

Vascular endothelial growth factor (VEGF) is an important pro-angiogenesis factor. Transplantation VEGF gene modified MSCs 24 hours after ischemia in rats, showed a significant functional recovery and lower infarct volume without aggravating cerebral edema comparing to the naïve MSCs-treated group [42]. Angiopoietin-1 (Ang1) gene modified MSCs and unmodified MSCs exhibited comparable reduction in brain lesion volume as compared with the control group. MSCs-Ang1 showed greater improvement of angiogenesis, neovascularization, regional cerebral blood flow, and functional recovery than the MSC cell therapy alone [43]. Some studies have combined two factors for gene modification MSCs to enhance the therapeutic effects of them for ischemic stroke. When combining Ang-1 gene and the VEGF gene to modify MSCs, rats that received MSCs-Ang-VEGF 6 h after MCAO showed the greatest structural-functional recovery as compared to the single gene modified MSC group [44].

In order to promote the recruitment of MSCs to the ischemic boundary, the receptor for the chemoattrative factor CXCL12, CXCR4 was also used to genetically modified MSCs. CXCR4 overexpression in the MSCs promoted their mobilization and enhanced neuroprotection in the rat cerebral ischemic model [45, 46]. Similar results were also observed in irradiated mice with transplantation of CXCR4 gene modified MSCs. The number of MSCs-CXCR4 homing to the bone marrow was highly correlated with plasma CXCL12 concentration [47]. In order to improve the survival rate of MSCs from oxidative stress, the frizzle-related protein 2 (sFRP2) gene was used to modify MSCs [48]. Gene modified MSCs have been shown to be a promising therapeutic treatment for ischemic stroke in animals. However, the clinical studies are still in its infancy. Many clinical trails and concerns call for more effort in this area.

18.2.2 Gene Modified Neural Stem Cells

Neural stem cells (NSCs) are defined as a kind of neural lineage stem cell that give rise to all types of mature neural cells including neurons, astrocytes, and oligodendrocytes [19, 49]. The first isolation of adult NSCs was reported in 1992 [50]. Embryonic NSCs can be expanded into neuroshperes, which are taken from the CNS of rodent embryos. These neurospheres are capable of self-renewal and differentiation into neurons, astroglial, and oligodendrocytes [51]. In the adult brain, NSCs gather in the subventricular zone (SVZ) and subgranular zone (SGZ). After stroke onset these endogenous NSCs can proliferate and migrate to the injured brain area, promoting brain repair [52, 53]. These data suggest that endogenous NSCs contribute to postischemic stroke repair. Unfortunately, the number of the endogenous NSCs is insufficient to complement the lost neurons, oligodendrocytes, and only a few newborn neurons were found in the brain [54]. NSC transplantation has been improved to enhance neurogenesis and is regarded as a promising therapeutic strategy for ischemic stroke [55, 56]. Overall, NSCs can be isolated from the CNS and expanded *in vitro* where they can be modified, then re-transplanted back into the brain so that they can integrate perfectly, differentiate into both neuronal and glial cells, and deliver exogenous genes into the brain. When human NSCs are injected into an early chronic injury microenvironment of immunodeficient NODscid mice, these cells can survive, differentiate, and promote locomotor recovery [19].

The extent of glial scar formation and the characteristics of inflammation are of the most remarkable differences in ischemic stroke between acute and chronic phases. The cytokines secreted during the acute phase, such as IL-1, IL-6, and CXCL1, might induce NSCs to differentiate into astrocytes. Furthermore, other growth factors like epidermal growth factor (EGF) and neurotrophin-3 (NT-3) have shown their beneficial effects on the function of NSCs [57, 58]. The differentiation properties of NSCs, their abilities to be genetically modified for transplantation, long-distance migration, and intergradation *in vivo* after transplantation can be employed for delivery and expression of therapeutic genes *in vivo* (Table 18.1).

Genetically modified NSCs have been shown to differentiate selectively into oligodendrocytes, which can remyelinate injured axons after transplantation in a demyelinated area [59]. Transplantation of nerve growth factor gene modified NSCs into the transient focal ischemic area in adult rats showed the ability to ameliorate the death of the striatum projection neurons [60]. Transplantation of VEGF overexpressed NSCs into intracerebral hemorrhagic stroke mice found that NSC-VEGF cells induced behavioral improvement, cell differentiation and survival, as well as renewed angiogenesis at 2 and 8 weeks post-transplantation [61]. In order to increase the differentiation of NSCs into neurons and oligodendrocytes after transplantation into brain, NT-3, known to play a role in inducing neuronal differentiation during development and following injury, was used to modify NSCs prior to transplantation into a postnatal day 7 mouse brain. NSC-NT-3 derived neurons increased to approximately 20% in the infarct cavity and >80% in the penumbra. These neurons were further differentiated into cholinergic, GABAergic, or glutamatergic subtypes. NSC-NT-3 derived glial cells were rare [62]. Following study showed that human NT-3 gene modified NSCs transplanted into the rat striatum after a severe focal ischemia promoted functional recovery compared to saline and naïve NSC treated animals [63]. To provide better therapeutic effects than GDNF or NSCs therapy alone on stroke, GDNF was used to genetically modify NSCs. After transplantation of NSC-GDNF cells into ischemic rats brain, there was a significant functional recovery and decreased lesion volume. GDNF modified NSCs also provided a better neuroprotection for stroke than NSC transplantation alone by increasing the NT-3 and BDNF protein [64]. Another study showed that BDNF modified NSCs in a rat model of cerebral ischemic stroke, showed a significantly lower neural severity score than the NSC transplantation alone and these cells differentiated into mature neurons 12 weeks after transplantation [65]. Akt-1, a kind of kinase protein, was also used to modify NSCs. Transplanted Akt-1-NSCs into ischemic animals showed an improvement in focal angiogenesis and neuronal survival in the brain [66]. These data suggest that NSCs and gene modified NSCs can be treated as effective candidates for ischemic stroke therapy, depending on better characterization of factors affecting NSCs.

18.2.3 Gene Modified Endothelial Progenitor Cells

Endothelial progenitor cells were first isolated from peripheral blood and reported in 1997. They were described as a population of bone marrow derived cells with CD34 positive cell surface marker [67]. EPCs present the ability to differentiate into endothelial cells involving into the vasculargenesis and angiogenesis [68, 69]. EPCs exhibit ubiquitously expressed markers and therefore EPCs lack a very definitive cell marker for characterization and isolation. However, there are some commonly used cell markers, such as CD31, CD34, KDR, and CD133, but these lack the expression of antigens like CD14, CD133, CD45, and CD115 [70–72]. EPCs have been showed to be incorporated into neovascularization *in vivo*, while transplantation of mature Ekes reportedly failed to get involved in revascularization in severely ischemic tissues [73, 74]. This kind of beneficial property of EPCs is attractive for cell therapy, as well as gene based stem cell therapy applications. However, the number of EPCs is so few in peripheral blood and bone marrow. Mobilization and the *ex vivo* explanation should be required for clinical applications [75, 76].

Genetically modifying EPCs by genes designed for growth and development may help maintain or enhance EPC function for therapeutic aims. Some factors were used to either enhance the biological functions of EPCs or to treat EPCs as vectors for gene therapy. This strategy can be applied to vascular injury diseases, such as vascular disease and ischemic diseases.

In myocardial infarct models, some factors genetically modified EPCs were used as a treatment, including insulin growth factor-1 (IGF-1), stromal derived factor-1 (SDF-1), endothelial NO synthase (eNOS), and deltalike4 (Dll4). IGF-1 modified EPCs showed an improved cardiac function and increased the number of capillaries in the periinfarct area in myocardial ischemia model [77]. Transplantation of SDF-1 gene transfected EPCs also showed a recovery of heart function and an augmentation of neovascularization after myocardial dysfunction [78]. eNOS expressing EPCs in the treatment of myocardial infarct animals exhibited a decreased inflammation response 3 days after transplantation. At 28 days after myocardial infarct, transplantation of EPC-eNOS cells improved cardiac function and reduced infarct size [79]. An angiogenic sprouting factor Dll4was also used to modify EPCs. When transplanted into a myocardial infarction model, EPC-Dll4 cells showed an increased mature vessles, blood flows, and an enhanced cardiac function [80].

Moreover, in a hindlimb ischemia model, VEGF, heme oxygenase-1 (HO-1), and hypoxia induced factor-1 (HIF-1) were employed for modifying EPCs. Transplantation of EPC-VEGF cells significantly increased the number of mature endothelial cells, blood perfusion level at 28 days after treatment compared to the bare EPCs [81]. Transplantation of co-transgender EPCs by VEGF and HO-1 into a rat hindlimb ischemic model showed ery significantly increased microvessel density, recovery of blood flow, and rate of limb salvage, compared with naïve EPC treated animals [82]. *Ex vivo* EPCs were modified by HIF-1, then transplanted into EPC-HIF-1 cells in nude mice with hindlimb ischemia. The mice receiving the EPC-HIF-1 cell transplantation showed a significant increased neovascularization and reduced necrosis at 14 days after transplantation [83].

In other kind of diseases, gene modified EPCs were also treated as a therapeutic strategy. After balloon-induced arterial injury in hypercholesterolemic rats, HGF gene modified EPC transplantation showed that the transfected HGF enhanced EPCs' homing ability to vascular injury site significantly decreased neointima formation and increased re-endothelialization [84]. Transplantation of VEGF modified EPCs into the corpora cavernosa of diabetic erectile rats showed an extensive neurovascularization in the corpora cavernosa and restored erectile function compared with the control group [85]. VEGF modified EPCs also showed its therapeutic function in treatment of ischemic surgical flaps, by increased capillary density and blood flow recovery compared to a non-modified EPC treated group [86]. Tissue plasminogen activator (tPA) engineered EPCs were employed to treat balloon-injured carotid arteries and attenuated reendothelialization of injured arteries [87]. Transplantation of human paraoxonase-1 (hPON1) gene modified EPCs into an atherosclerosis model showed a more satisfactory therapeutic effect than the control group [88].

Few researches were done in experimental ischemic stroke (Table 18.1). CXCR4 primed EPCs in the treatment of diabetic ischemic mice models showed enhanced efficacy of EPC infusion in attenuating infarct volume and promoting angiogenesis and neurogenesis [89]. More work should also be done by using gene modified EPCs as a treatment for experimental ischemic stroke.

18.2.4 Induced Pluripotent Stem Cells

The induced pluripotent stem cell (iPS) was first produced from a nonpluripotent mouse cell in 2006, when it was first realized that mature cells could be reprogrammed to become pluripotent cells [90]. iPS cells are typically reprogrammed from adult mice somatic cells by inducing forced expression of four transcription factors: Oct4, Sox2, c-Myc, and Klf4 [90]. iPS cells exhibit similar properties to ESCs, expressing typical ESC cell specific markers and giving rise to multiple lineages *in vitro*, which make iPSs a kind of promising stem cell [91]. Consequently, successful iPS cells were obtained from human fibroblasts both by retroviral transfection of Oct3/4, Sox2, Klf4, c-Myc genes, or Oct4, Sox2, and Nanog Lin28 genes in 2007 [11, 92]. A number of studies showed that iPS formed a teretoma during its migration and maturation into neurons in the ischemic region [93]. Since then, different transfection tools have been used to modify transfection efficiency and avoid the risk of teratogensis [94, 95].

iPS cells overcome the ethical and immunological concerns raised by using ESCs [96]. iPS can be derived from a patient's own somatic cells [97]. Some studies have demonstrated that iPS cells could specifically differentiate into glutamatergic neurons, motor neurons, and GABAergic neurons [98]. These cells are very helpful in treatment of stroke. Intracerebrally grafted iPS cells into the ischemic brain showed a migration to the ischemic region, differentiation into neurons, reduction of infarct volume, and improvement of functional recovery [99, 100]. Studies have also showed that iPS-derived NSCs are far more safe and effective than iPS cells for the treatment of cerebral ischemic stroke. iPS-derived NSCs presented a cortical cell phenotype and electrophysiological property of mature neurons, integrating into the host circuitry in a mouse with no teratoma formation [101]. iPS-derived NSCs migrated in the perifocal region, and matured into neurons and astrocytes, which results in neurological functional recovery in a rat model [102]. The discovery of iPS has opened a huge new potential therapeutic approach for ischemic stroke.

18.3 Gene Transfer Vectors

In order to successfully modify stem cells, many tools have been used to transfect them, which normally include viral vectors and non-viral vectors. Many viral vectors, such as baculovirus, retrovirus, lentivirus, adenovirus, and adeno-associated virus, have been developed for gene modification depending on the types of stem cells and gene expression time course. Non-virual vectors, including plasmids and other materials, are developed to avoid the side-effects of virus vectors and other kinds of necessary aims. The aim of gene delivery into stem cells is to gain safe and effective modified stem cells. Naked DNA delivery without vectors is a very difficult method for penetrating most cell membranes. Both viral and non-virual vectors here are to help achieve safe and efficient for gene delivery. The viral vector delivers the gene into the stem cells as part of their replication cycle, while the non-viral chemical vector generally penetrates the cell via endocytic uptake [103].

18.3.1 Viral Vectors

With regards to infect efficiency, the viral vector that contains genes is the most powerful tool. The viral system can be separated into two groups: integrative and episomal formation. Retrovirus-based vectors, including lentivirus, are able to achieve stable transgene expression by integrating the exogenous gene into the host genomes. This kind of vector exhibits highly efficient transduction and stable gene expression in

proliferating and non-proliferating cells. However, there exists a risk of insertional mutagenesis, which may lead to tumorgenesis. Retrovirus can permanently integrate the transgene into the chromosome of the target cell [104]. However, the low titer of retrovirus limits its use in stable gene transfer studies. Another member of the retrovirus family, lentivirus, has shown high transduction efficiency in tissue-derived stem cells and long-term expression [105]. Adeno-associated virus (AAV), unlike retrovirus and lentivirus, retains the ability of site-specific integration into the transcriptionally silent AAVS2 region of human chromosome 19 (position 19q13.42). Therefore, AAV leads to safer application and lower risk of mutagenesis. These kinds of integrating viral systems have a capacity of about 4–8 kb, a relatively low capacity, which limits the viral genome construction [106]. Balculovirus (BV), with a high cloning capacity of about 38 kp, facilitates the accommodation and delivery of a large functional gene construct. Due to the properties of nonintegration and nonreplication of BV, it induces only transient gene expression, approximately less than 7 days [107]. hMSCs can be genetically engineered by the baculovirus-containing morphogenetic protein-2 (BMP-2) for an efficient cell differentiation into osteoblasts, which was shown to help bone formation [108]. Adenoviral vectors usually cannot integrate into the cellular genomic DNA and infect both proliferating and non-proliferating cells. However, adenovirus can cause humoral and cellular immune reactions in host cells. Other viral vectors, such as retrovirus, lentivirus, and adeno-associated virus, ensure permanent gene expression, which can help the gene's long-term expression in stem cells, but increases the risk of insertional mutagenesis and is only used in experimental research. A fiber-modified adenovirus, loaded with Kringle1–5 gene, an angiogenic inhibitor, was also used to transfect human tissue-derived MSCs. The transfection efficiency can be up to 90% and an effect was found on VEGF expression blocking [109]. Different virus vectors present their own merits and pitfalls; care should be taken when we choose the types of virus for stem cell modification.

18.3.2 Non-Viral Vectors

Besides viral vectors, there is also another group of transfer vehicles; non-viral vectors. When compared to viral vectors there are some advantages of non-viral vectors, such as broad production and little host immunogenicity [110]. Physical delivery transformation is usually enforced by external forces, such as electrical and magnetic tools. The commonly used non-viral vectors are chemical vectors, such as cationic lipids, polymers, inorganics, and composites [111]. Although many modifications have been made to modify chemical vectors, the transfection efficiency for stem cells is also a major hurdle. Here, we give some examples of chemical vectors that have the capacity to transfect stem cells with a lower toxicity.

By combination of an extracellular matrix-like scaffold with therapeutic molecules, specific DNAs can be used for stem cell transfection. A porous sponge-like collagen scaffold for non-viral delivery of plasmid encoding for GDNF to rat marrow stromal stem cells, with a proper ratio of plasmid to lipid transfection reagent (2:1) and cell density (initially seeded with 2 million cells in the plate), accumulated GDNF 60% greater after 9 days of culture. This may be a promising non-viral approach for the long-term production of neurotrophic factors in MSCs in general [112]. The EphrinB2 gene was successfully transfected into rat MSCs by polyethyleneimine (PEI), a non-viral vector [113]. Using a cationic liposome-based reagent, plasmid DNA delivery was optimized for safe and transient expression in MSCs. When using a lipid/DNA ratio of 1.25 after transfection for 7 days, the transfection efficiencies ranged approximately 2–35% and the transfected cells presented high viabilities (>90%) and recoveries (>52%) [114]. A diblock copolyer was examined for delivering siRNA to MSCs. The diblock copolymers consisted of two kinds of blocks; cationic blocks, which were capable of siRNA dispersion, protection, and uptake; the other was pH-sensitive blocks, which prohibited indusial escape. The diblock copolymers were shown to facilitate nearly 100% of MSC uptake of siRNA and knocked down control gene expression to 10% [115]. A multi-modal (MR/optical) transfect ion agent (MTA) for both efficient gene delivery and cell tacking was used to transfect MSCs. Cationic poly-immobilized MTAs were capable of binding DNA molecules for successful MSCs transfection and were visualized after transplantation via MR and optical imaging system over 14 days [116]. Another

type of biodegradable polymeric vector was also developed for non-viral gene transfer in human stem cell lines, including MSCs, adipose-derived stem cells (ADSCs), and human embryonic stem cell-derived cells (ESCds). Culturing with 10% serum, leading end-modified C32 polymeric vectors can highly transfect these three kinds of cells, all providing high cell viability [117].

Although non-viral vectors for MSCs transfection have been widely developed, there are still many other types of stem cell that need to be elucidated, such as neural stem cells, endothelial progenitors, iPS, and so on. Depending on the different DNAs or siRNAs for different stem cells, specific transfection vectors still need to be optimized.

18.4 Unsolved Issues for Gene-Modified Stem Cells in Ischemic Stroke

Before stem cell based gene therapies become a routine and clinical treatment, there are still many issues to be solved. These issues include:

1. What types of stem cells, source, and quality control when expanded *ex vivo* make the best choices for stem cell-based gene therapy?
2. Which kind of transgenic vector is the most optimized vector? Viral vectors are more efficient and of longer duration than non-viral ones, but with more concerns about toxicity, immune response, inflammation, and insertional mutagenesis.
3. Which gene should be used to modify stem cells when considering a special stem cell or special patients?
4. What kind of patient should be involved in stem cell based gene therapy?
5. How many cells should be given to patients?
6. What is the best treatment window after ischemic stroke?
7. Which routes are the best delivery routes; intravenous, intracerebral, or intraventricular?
8. What are the monitoring standards for evaluation of patients' therapeutic progression?

18.5 Conclusion

The development of stem cell and gene therapy has provided fundamental research for stem cell based gene therapy in ischemic stroke. Stem cell based gene therapy has become an area of vital importance. Stem cells provide self-renewing and differentiating gene carriers. In turn, the transgene helps stem cells with better survival and differentiational abilities. Meanwhile, genetically modified stem cells also exhibit antocrine and paracrine effects for ischemic stroke treatment by anti-inflammation or neuro-protection. More vectors, including viral and non-viral, aim to promote transgene efficiency and safety, and reduce toxicity and tumorgenesis. The overall goal for gene modified stem cells is to provide sufficient treatment methods for ischemic stroke. Even though many hurdles still exist, we predict a very promising future for gene modified stem cells for ischemic stroke treatment.

Abbreviations

AAV	Adeno-associated virus
ADSCs	Adipose-derived stem cells
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor

BV	Balculovirus
Dll4	Deltalike4
EGF	Epidermal growth factor
eNOS	Sndothelial NO synthase
ESCds	Human embryonic stem cell-derived cells
ESCs	Embryonic stem cells
GDNF	Glial cell line-derived neurotrophic factor
HGF	Hepatocyte growth factor
HIF-1	Hypoxia induced factor-1
HO-1	Heme oxygenase-1
IGF-1	Insulin growth factor-1
iPS	Induced pluripotent stem cell
MCAO	Middle cerebral artery occlusion
MSCs	Mesenchymal stem cells
NSCs	Neural stem cells
PIGF	Placenta growth factor
SDF-1	Stromal derived factor-1
SGZ	Subgranular zone
SVZ	Subventricular zone
tPA	Tissue plasminogen activator
VEGF	Vascular endothelial growth factor

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19

Role of Stem Cells in the Gastrointestinal Tract and in the Development of Cancer

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19.1 Introduction

The gastrointestinal (GI) tract is a group of developmentally related organs responsible for digestion, absorption, detoxification, and excretion. The GI system includes the luminal gut and gut derived organs, such as the esophagus, stomach, small intestines, large intestines, pancreas, and liver. GI diseases often lead to poor prognoses and high mortality. For instance, two of the top three most dangerous cancers with the highest mortalities, pancreatic, and colon cancer, occur in the GI tract. According to a report by the World Health Organization (WHO), GI malignant neoplasms, such as liver cancer, colon cancer, stomach cancer, pancreatic cancer, esophageal cancer, mouth, and oropharyngeal cancers caused 457 deaths per million of the population worldwide in 2012. Other GI system-related diseases, including digestive diseases and hepatitis virus infection, caused additional 346 deaths per million population worldwide in 2012 (WHO 2015). Genetic, environmental, and epigenetic factors contribute to the development of GI-related diseases.

Among risk factors for developing GI diseases, environmental factors, especially environmental toxicants, contribute to a significant number of cases. Exposure to irradiation and heavy metals cause direct DNA damage, which is an important factor for the development of cancer. Excess exposure to toxicants can cause direct tissue injuries, genetic mutations and epigenetic modifications. Overdoses of drugs, such as paracetamol

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and acetaminophen, often induce hepatotoxicity and contribute to 39% of acute liver failures in the USA (Ostapowicz, et al. 2002). Abuse of alcohol also leads to liver injury, such as steatosis, hepatitis, and cirrhosis (O'Shea, et al. 2010). One to two percent of patients with alcoholic liver cirrhosis develop hepatocellular carcinoma (HCC) per year (Verma 2009). Another important risk factor for developing cancer of the GI system is tobacco use. Tobacco contains over 60 carcinogenic components, such as nicotine, polycyclic aromatic hydrocarbons, and nitrosamines. These carcinogens can act as mutagens leading to DNA misrepair, chromosomal instability, and epigenetic modifications. A recent study showed that newborn infants exposed to maternal tobacco use have significantly different methylated DNA in cord blood when compared to normal infants (Ivorra, et al. 2015). During the development of cancer, some of these carcinogens, such as nicotine, can also act as mitogens. Cigarette smoking is listed as a major risk factor for oropharyngeal and esophageal cancer. Increasing evidence also shows a strong association between tobacco use and many other GI system cancers, including stomach cancer, HCC, and pancreatic cancer (Das 2003, Shin and Cho 2005).

In GI cancers, certain stem cells contribute to proliferation and metastasis. Stem cells are able to divide by self-renewal in somatic tissues. There are three types of stem cells: totipotent cells such as germ line stem cells, pluripotent stem cells such as embryonic stem cells (ES) and induced pluripotent stem cells (iPSc), and multipotent stem cells such as somatic stem cells. ES or iPS cells, which are capable of differentiating into three germ layers (Yamanaka, et al. 2008), have been induced into terminally differentiated tissues including those of the GI tract. Compensatory activation of somatic stem cells usually occurs after tissue injury caused by mechanical damage or toxicity. Somatic stem cells, also known as adult stem cells, are capable of self-renewal and generating certain specialized cell types. These cells are usually relatively small in population and remain quiescent until activated for maintenance and repair of tissue. Generally speaking, somatic stem cells can only differentiate into limited cell types within an organ. Maintenance and regulation of somatic stem cells require a certain environment called a "stem cell niche." Aberrant alterations of the niche can lead to differentiation, transdifferentiation, cell death, and carcinogenesis.

Stem cells play key roles in development, tissue homeostasis, and regeneration following injury in adult tissues. Moreover, stem cells have been experimentally shown to be the cells of origin for certain types of cancer. To understand the biology of stem cells and to exploit their use in therapeutics, it is critical to identify and characterize the factors that control the decision between self-renewal and differentiation under normal physiological conditions and in disease. Studies on stem cells can help us to understand critical events during development and stem cell differentiation towards a functional somatic cell fate, as well as tumor initiation. Using flow cytometric sorting and single cell-based assays, hepatic stem cells in rodent (Suzuki, et al. 2000, 2002, Zheng, et al. 2014) and human fetal livers (Zhang, et al. 2015) have been identified. These cells can differentiate *in vitro* as well as *in vivo*. The isolated fetal hepatic stem cells are also characterized by the ability to maintain their self-renewal capability in the developing liver. Cancer stem cells appear to share important functions with normal stem cells, such as self-renewal, proliferation, and long-term survival. It is therefore believed that a similar set of critical genes control both normal and cancer stem cells. The existence of cancer stem cells is of great clinical relevance since their unique stem cell properties are likely enabling them to escape conventional anti-cancer therapeutics designed to target the fast cycling and highly proliferating cancer cells. Cancer cell resistance to chemoradiotherapy is hindering treatment efforts in clinics. Thus, prevention strategies and early detection may reduce mortality. We have proposed the concept of using precancerous cells and their progeny in cancer therapy (Zheng, et al. 2012), which could provide unique advantages for early cancer diagnosis, treatment, and preventive therapy. In addition to discussing the nature and characteristics of precancerous cells and their progeny, we have also introduced an effective precancerous cell-targeted therapy based on an animal model of HCC (Zheng, et al. 2014). Anti-precancerous cell drug development should be a major focus for elimination of cancers, and it may lead to preventive therapies for individuals with a high risk of developing cancer. Our focus on liver disease has led to progress in the fields of hepatic stem cells, cancer stem cells, and tissue regeneration. This chapter will

cover topics ranging from the fundamental properties and comprehensive descriptions of the development, regeneration of the GI tract and GI stem cells to epigenetic changes and reprogramming.

19.2 GI Development and Regeneration

19.2.1 GI Development

Somatic cell development is a programmed process controlled by external signals, internal genetic information, and epigenetic modifications. During the process of GI development, the primitive streak undergoes epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition to form the endoderm, in which signaling pathways such as Wingless-type (Wnts), bone morphogenetic protein (BMP), and fibroblast growth factor (FGF) are involved. The liver arises from the ventral foregut endodermal epithelium. During endoderm development, the ventral foregut condenses to form the definitive endoderm, which will develop into the liver and pancreas (Zaret and Grompe 2008). During the development from definitive endoderm to hepatocytes and cholangiocytes, many transcription factors are involved. Forkhead box A proteins (Foxa1, Foxa2, and possibly Foxa3) function redundantly for liver gene expression (Zaret 2008). GATA-binding proteins (Gata4 and Gata6) are also redundantly necessary for liver development, though neither of them is required for liver fate specification (Zaret 2008). FoxA and Gata4 function as pioneer factors to open compacted chromatin and make local chromatin accessible to additional transcription factors, which are required for additional signaling to initiate the liver gene program (Cirillo, et al. 2002). Such additional signaling includes FGF and BMP signaling from adjacent mesodermal tissues (Jung, et al. 1999, Rossi, et al. 2001). FGF is particularly important for determining liver fate versus in the pancreas (Deutsch, et al. 2001).

After specification of liver fate, hepatoblasts undergo differentiation towards either the hepatocyte or biliary lineage. Hnf1β and Hnf6 are responsible for biliary specification. Deficiency of either Hnf1β or Hnf6 results in dysplasia of the bile ducts (Clotman, et al. 2002, Coffinier, et al. 2002). Hnf4α and Hnf1α are also important for the differentiation of hepatocytes. Homozygous Hnf4α deficiency causes a defect in mature hepatocyte gene expression (Li, et al. 2000). Hnf1α binds to many hepatic promoters and regulates the expression of mature hepatocyte genes.

It is interesting that none of the hepatic transcription factors mentioned here are exclusively expressed in hepatocytes. For example, the FoxA family genes are also required for the development of the pancreas, lung, prostate, and kidney (Friedman and Kaestner 2006). Developmentally related tissues, such as the liver and pancreas, often use similar sets of transcription factors to specify different lineage. Hepatic transcription factors, such as FoxA, Gata4, Hnf1α, and Hnf4α, also regulate pancreatic genes. An explanation for the different responses to the same set of transcription factors lies in the different chromatin prepatterning of tissues (Xu, et al. 2011).

Systematic analysis found that hepatic transcription factors cross-regulate their own and each other's expression by binding to promoters (Kyrmizi, et al. 2006). The cross-regulation circuit stabilizes hepatic cell fate. Activation of part of the transcription factors can reestablish the cross-regulation circuit (Huang, et al. 2011, 2014, Sekiya and Suzuki 2011) or defined as reprogramming.

19.2.2 GI Stem Cells and Liver Regeneration

Somatic stem cells can be derived from fetal, postnatal, or adult tissue. In the fetal liver, Suzuki et al. reported that by using fluorescence-activated cell sorting (FACS), they found that $CD49f^{+}CD29^{+}c\text{-}Kit^{-}CD45^{-}Ter119^{-}$ cells from the fetal liver can form colonies *in vitro*, and moreover, these cells are bipotent

and could differentiate into hepatocytes and cholangiocytes *in vitro* (Suzuki, et al. 2000, 2002), indicating the progenitor/stem cell capability of these cells. Moreover, hepatic progenitor cells were also found in the normal adult liver with FACS (Dorrell, et al. 2011, Suzuki, et al. 2008). In adult tissue, the existence of somatic stem cells in the GI tract was first postulated because of the incapacity of terminally differentiated GI cells to replicate. Over time, a number of somatic stem cells have been characterized in the GI system. In the intestines, the stem cell niche is found at the crypt base (Barker 2014). $Lgr5^+$ crypt base columnar cells were then identified as the intestinal stem cells responsible for the integrity and homeostasis of the small intestines and colon (Barker, et al. 2007). As a Wnt signaling pathway member, Lgr5 also marks Wnt-regulated somatic stem cells in many other GI tissues, including the stomach and liver (Huch, et al. 2013, 2015). However, the existence of stem cells in the liver is still controversial. First, Lgr5 does not exist in the normal mouse liver and is only found in injured liver biliary cells. Second, most hepatocytes maintain the ability to replicate. Recent reports indicate that hepatocytes themselves are the hepatic stem cell in the normal (Font-Burgada, et al. 2015, Wang, et al. 2015) and injured liver (Tarlow, et al. 2014, Yimlamai, et al. 2014), no matter where the cells are located. In most liver injury animal models, liver regeneration is mostly completed by hepatocyte replication (Stocker and Heine 1971). Liver stem cells often arise in widespread hepatocyte lesions, though the contribution of liver stem cells in liver regeneration is relatively limited (Grompe 2014).

The liver is the largest organ within the human body and is capable of robust regeneration in adult humans. The process of liver regeneration has been comprehensively examined, though the detailed mechanism still largely remains unclear. Liver regeneration can be induced by numerous stimuli, including mechanical injuries, toxification, viral infection, and other liver diseases. Though many signaling pathways, such as IL-6, EGF, and HGF, are activated both in liver regeneration and tumorigenesis, the well-organized proliferation of liver cells and arrangement of new cells makes liver regeneration different from tumorigenesis (Fausto, et al. 2006). Most of our knowledge of liver regeneration is from the use of partial hepatectomy (PHx), which is valued for its repeatability and well-defined stimulation of liver regeneration. In an adult body, a normal liver can regenerate and recover itself after PHx. In the murine liver, PHx triggers the destruction of the extracellular matrix (ECM), which secretes cytokines to induce hepatocyte proliferation. Numerous studies suggest that ECM degradation induces hepatocytes to enter a priming state that is ready to respond to mitogens, though it is still unclear how mechanosensing triggers proliferation of liver cells. Within 30 min after PHx, the ECM starts to degrade (Kim, et al. 2000, Mars, et al. 1995). Degradation of the ECM releases growth factors, such as HGF, and induces mechanical distortion (Mars, et al. 1993, Naldini, et al. 1995). Hepatocytes proliferate at a rate that peaks at 24 h (Michalopoulos and DeFrances 1997). The cells begin to communicate with other hepatocytes and non-parenchymal cells in the liver via paracrine and endocrine signaling pathways. After 24 h, non-parenchymal cells are activated and begin to proliferate. Several cytokines have been found to be involved in the induction of liver regeneration. HGF, previously stored in the ECM or newly synthesized by Ito cells (hepatic stellate cells), is a potent mitogen for hepatocytes both in cell culture and in vivo after PHx (Nakamura, et al. 1989, Zarnegar, et al. 1991). EGF and TGFα may also play critical roles in the promotion of liver regeneration. However, direct injection of HGF and EGF show relatively minor effects on DNA synthesis of hepatocytes, suggesting induction of the priming state is needed before mitogens can function (Roos, et al. 1995, Webber, et al. 1994). Moreover, TGFα deficient mice show normal liver regeneration (Russell, et al. 1996). The reason for that might be the compensatory functions of other growth factors. Deficiency of either TNFα type I receptor or IL-6 suppresses liver regeneration (Cressman, et al. 1996, Yamada, et al. 1997). The growth factors mentioned above significantly increase DNA synthesis of hepatocytes in culture. The murine liver mass restores itself back to the normal state after PHx within 1 week, followed by the termination of liver regeneration. During this termination process, signals inducing apoptosis such as TGFβ are involved. Continuation of liver regeneration without termination

will cause abnormal liver formations such as liver fibrosis. However, continuous proliferation and large-scale expansion of hepatocytes in cell culture are still big challenges.

Many pathways regulate GI organ size. Among these, the hippo pathway is found in *Drosophila* to control organ size (Yu, et al. 2015). Mutations in the hippo pathway induce overgrowth of liver, intestines, kidney, lung, stomach, and pancreas, not only in *Drosophila*, but also in mice (Yu, et al. 2015). Chemical compounds, such as verteporfin and metformin targeting the hippo pathway can then inhibit the tissue overgrowth or cancer (Yu, et al. 2015).

The liver is a major organ participating in food metabolism, defense against toxins, alcohol processing, and pharmaceutical metabolism. Nutrients and detoxicants are transported by the liver ducts to the blood and intestines, and during these processes, the liver is exposed to many injuries and consequently, liver repair or regeneration.

As described previously, hepatocytes retain the ability to proliferate *in vivo*, and they play a major part in liver regeneration after PHx. Serial transplantation of hepatocytes into an injured mouse liver revealed the stem cell capacity of mature hepatocytes (Overturf, et al. 1997). Growing evidence suggests that the homeostasis of liver mass is maintained by replication of preexisting hepatocytes rather than differentiation of liver stem/progenitor cells in normal adult livers (Malato, et al. 2011, Yanger, et al. 2014). Pericentral $Axin2^+$ hepatocytes proliferate faster than liver stem/progenitor cells, and thus, contribute to the homeostasis of liver mass as an important source of hepatocytes (Wang, et al. 2015).

Liver regeneration after injury is more complex. The role of liver stem/progenitor cells during liver regeneration is still controversial. The most commonly used liver regeneration model is 70% PHx. After 70% PHx, most mature hepatocytes reenter the cell cycle and contribute to liver regeneration, suggesting mature hepatocytes as the major contributor to liver restoration (Stocker and Heine 1971). However, in some toxic chemical-induced chronic liver injury mouse models, bipotential liver stem/progenitor cells (oval cells) arise in the canals of Hering, between the hepatocytes and bile ducts. These oval cells are capable of differentiation into both hepatocytes and cholangiocytes, though they only contribute to new hepatocytes (Schaub, et al. 2014, Tarlow, et al. 2014). A new cluster of liver stem cells expressing Lgr5 was also identified in toxic chemical-treated livers. $Lgr5^+$ liver stem cells can form organoids in 3D culture and differentiate into hepatocytes *in vitro* (Huch, et al. 2013).

Lineage tracing showed that the so-called bipotential liver progenitors are dedifferentiated from mature hepatocytes after chronic injuries (Tarlow, et al. 2014). In addition, recent studies found that mature hepatocytes, especially hybrid periportal hepatocytes that express both hepatic and bile duct genes, are the major source of hepatocytes responsible for liver restoration after chronic liver injuries (Font-Burgada, et al. 2015). Hence, this topic is still under debate and more evidence should be gathered to address whether either hepatocytes or duct cells in the biliary tree have a stem cell-like function in the normal liver and during injured liver regeneration.

19.3 GI Tumorigenesis and Stemness Gene Expression

Gastroenterological cancers, including pancreatic, liver, gastric, and colorectal cancer, are among the most common malignant cancer types. They are similar in that they are epithelial cell-derived tumors and are invasive and migrate to other sites. Gastroenterological tumor cells are highly heterogeneous. A population within the tumor possessing the ability to give rise to all tumor cell types has been identified as tumor initiating cells (TIC) or cancer stem cells (CSCs) for many solid malignancies. The origin of CSCs is still controversial. In normal tissues, continuous division of somatic stem/progenitor cells maintains tissue mass, which also makes somatic stem/progenitor cells more likely to accumulate mutations, and thus, more likely to transform. One such example is transformation of human breast stem cells leading to breast

carcinoma (Ince, et al. 2007). Overexpression of genes related to the self-renewal capability of hepatic stem cells also transforms normal stem cells into CSCs (Chiba, et al. 2007). Thus transformation of somatic stem cells has been considered to be a source of CSCs (Hartwig, et al. 2014, Reya, et al. 2001). Another possible source of CSCs is differentiated somatic cells. By expression of four pluripotent transcription factors, somatic cells can be reprogrammed into pluripotent stem cells (Takahashi and Yamanaka 2006). In normal tissues, especially in the GI tract, interconversion of stem cells and differentiated cells is also found with certain conditions. Intestinal $Dll1^+$ secretory progenitor cells can be converted to stem cells after ablation of Lgr5 stem cells caused by radiation damage (van Es, et al. 2012). Recently studies also found that mature hepatocytes can give rise to liver stem cells and cholangiocytes after liver injury (Dorrell, et al. 2014, Tarlow, et al. 2014). Thus, it is not surprising that differentiated cells may also give rise to CSCs. There is plenty of evidence showing that CSCs can also originate from non-tumor initiating cancer cells. In an inducible model of breast cancer, mammary epithelial cells were induced to transform and became non-stem cancer cells. Additional IL6 signals thus contribute to conversion of breast non-stem cancer cells to CSCs (Iliopoulos, et al. 2011).

Most somatic cells retain their whole genome and exhibit plasticity. Overexpression of lineage specific genes is able to reprogram the epigenome and transcriptome of somatic cells, thus inducing cell fate conversion. In addition, cell fate conversion can be induced by modification of cell culture media, indicating that changing of certain cell signals is capable of resetting the whole epigenome and transcriptome (Hou, et al. 2013). Tumorigenesis, another form of cell fate conversion, shares multiple regulation mechanisms with reprogramming. EMT, which is found in cancer metastasis, is also shared by normal stem cells (Li, et al. 2011, Mani, et al. 2008), indicating the stem cell-like property of plasticity tumor cells. Overexpression of EMT regulators, such as Slug, would reprogram somatic cells into tumor initiating cells (Guo, et al. 2012). Thus, EMT-associated genes such as Snail, Twist, and Slug have been used as tumor indicators.

Tumorigenesis often results from DNA mutations caused by environmental factors. 2-Acetylaminofluorene (2AAF), a carcinogenic and mutagenic chemical that causes DNA damage, and PHx are used to study rat liver carcinogenesis (Zheng, et al. 2014). Oval cells, a tumor-initiating cell (TIC) in the liver, can be isolated using the cell surface markers CD133 and CD44 (Zheng, et al. 2014). CD133 and CD44 have been used to identify cancer stem cells in gastric tissue including the liver (Ma 2013, Takaishi, et al. 2009). Moreover, a drug that specifically targets liver tumors also inhibits oval cell expansion and induces oval cell differentiation *in vitro* and *in vivo*. Another report showed evidence that antibodies against CD47, which is a leukemia stem cell marker also found in gastric cancer, could treat the patient by increasing the survival rate (Chan, et al. 2009). Thus, it is possible that therapeutics targeting TICs can inhibit tumor growth in patients.

19.4 Toxicants and Other Stress Trigger Epigenetic Changes, Dedifferentiation, and Carcinogenesis

Toxic chemicals have been found to modify cell fate by the induction of epigenetic changes. During the 1980s, several groups induced hepatic cells in the pancreas, especially after treatment with toxic chemicals such as NBOP and ciprofibrate (Reddy, et al. 1984, Scarpelli and Rao 1981). An *in vitro* study with the pancreatic cell line AR42J-B13 showed evidence of direct transdifferentiation of AR42J-B13 cells to hepatocyte-like cells after treatment with dexamethasone (Shen, et al. 2000). During liver regeneration, hepatocyte apoptosis is induced by TGFβ, and moreover, TGFβ has been found to induce hepatocyte EMT (Oberhammer, et al. 1992, Sheahan, et al. 2008). Other liver carcinogenetic chemicals, such as diethylnitrosamine (DEN) and phenobarbital, aflatoxin, and thioacetamide as well as carbon tetrachloride (CCl_4) and 2AAF, are widely used to induce murine liver cancer (Heindryckx, et al. 2009, Zheng, et al. 2014).

A choline deficient diet, a cholangiocyte proliferation inducer, also causes liver cancer (Heindryckx, et al. 2009). Hepatic stem cells are induced by CCl_4 and with the choline deficient diet in liver injury models (Huch, et al. 2013). Forced expression of transcription factors, in addition to chemicals, can also induce hepatocytes into pluripotent stem cells (Imamura, et al. 2010), indicating the plasticity of hepatocytes. Moreover, Chiba et al. reported that overexpression of polycomb-group gene Bmi1 and β-catenin in the liver significantly induces the development of hepatocytes into cancer stem cells (Chiba, et al. 2007, 2008).

Somatic stem cells are usually activated after loss of differentiated cells in order to maintain tissue mass. Toxicants are among the risk factors for differentiated cell loss. Toxicant-induced liver injury accounts for nearly half of the cases of acute liver failure (Kaplowitz 2004). Many toxicants, such as CCl_4 and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), as well as a methionine choline-deficient ethionine-supplemented (MCDE) diet induce massive liver injury, which leads to appearance of bipotential liver stem/progenitor cells (Dorrell, et al. 2011). However, recent studies have shown that liver stem/progenitor cells are derived from dedifferentiation of hepatocytes (Dorrell, et al. 2014, Schaub, et al. 2014, Tarlow, et al. 2014). A similar process also occurs during the development of liver cancer stem cells (Yamashita and Wang 2013).

Unlike the programmed differentiation of stem cells, cell fate conversion is usually the result of reprogramming. Environmental toxicants often act as epigenetic modifiers, which sometimes cause sporadic reprogramming. One such example is hepatic fate induced in pancreatic cells by dexamethasone (Shen, et al. 2000). Long-term exposure to toxic chemicals may even cause carcinogenesis, a kind of malignant cell fate conversion. For example, trichloroethylene (TCE), a common used industrial solvent, may induce the expression of the oncogenes c-Jun and c-Myc in the liver by reducing the methylation of their promoter regions (Tao, et al. 1999). As we described here for the liver, mature cells in the GI tract are plastic and are capable of dedifferentiating into progenitor cells under epigenetic change.

19.5 Summary and Perspective

We have discussed the relationships between stem cells and cancers in the GI tract, as well as chemicals involved in these events. Increasing evidence shows that the GI tract has undergone comprehensive analyses, however, the effects of toxic chemicals on GI organs and stem cells are still not fully understood. Under damaging conditions, cells struggle to find a stable state in order to maintain their internal environment, acquiring migration characteristics and adjusting to their microenvironment. Most cells cannot survive under such circumstances with extra stress, toxicants, abnormal stimuli, and nonphysiological surroundings. The concept that stress is an important factor for inducing stem cells is promising. The schematic process from the accumulation of stress to the gain of stemness is summarized in Figure 19.1. It is hypothesized that the transformation of normal somatic cells to cells with plasticity is accompanied by key cellular epigenetic changes or barriers during this process. To learn more about the stress to stem cell conversion, single cell genomic sequencing is under further investigation. In clinical practice, due to the high mortality associated with GI cancers, further studies should focus on non-invasive diagnosis or prediction for the cancers, such as testing circulating cells in the patients.

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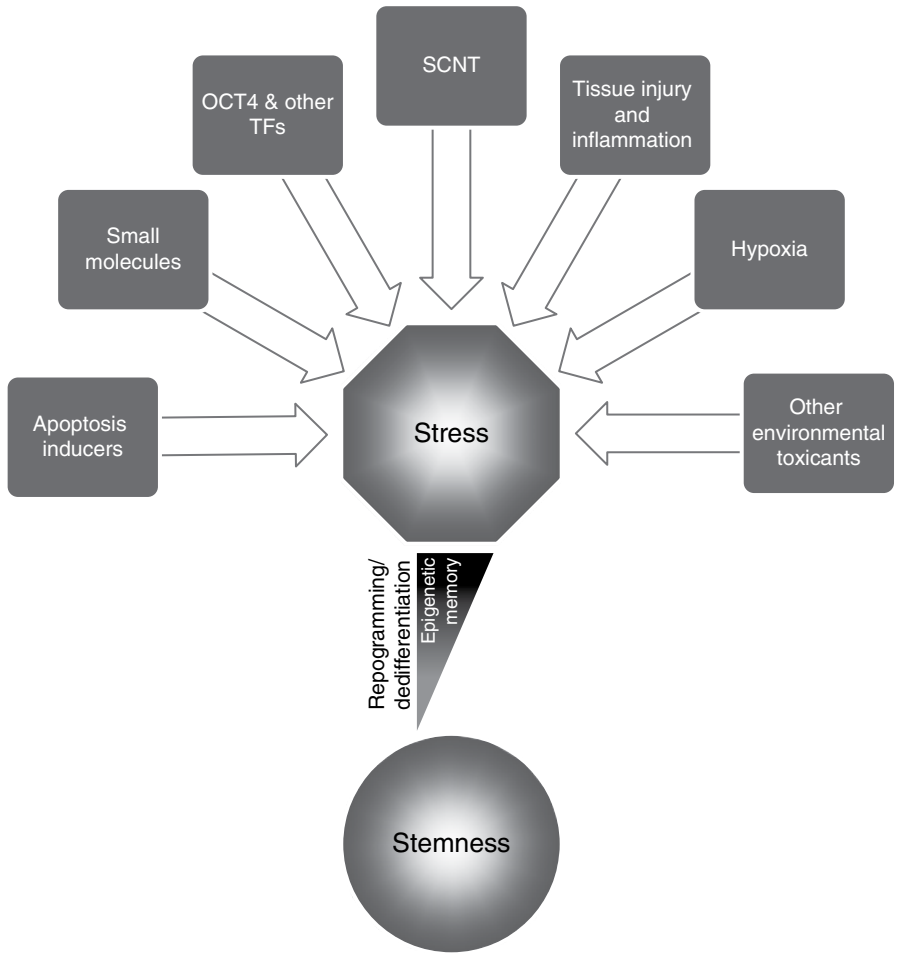

Figure 19.1 *The process of cellular dedifferentiation and reprogramming from somatic cells to stem cells. When the cell accumulates stress including stimulation by somatic cell nuclear transfer (SCNT), transcription factors (TFs) such as Octamer-binding transcription factor 4 (OCT4), etc., chemicals, small molecules, tissue injury and repeated inflammation, apoptosis inducers, and/or hypoxic microenvironment. This results in demethylation, epigenetic barriers, or memory erasing, and finally triggers the dedifferentiation and partial or full reprogramming of the cell*

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20

Cancer Stem Cells: Concept, Significance, and Management

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20.1 Introduction

Cancer is the second leading cause of disease-related deaths worldwide. According to an estimate, approximately 638,910 people are diagnosed with this disease every year in the United States alone and nearly 577,190 die due to it (Siegel et al., 2016). Cancer initiation and development is a multistep process requiring progressive clonal selection of transformed cells with enhanced tumorigenic ability. Cancer is a heterogeneous mass of abnormal cells of human body with uncontrolled cellular functions such as proliferation and resistance to apoptosis. These cells subsequently invade locally and metastasize to distant organs. The heterogeneity within the tumor cells results from a wide range of phenotypic, genotypic, and morphological differences (Chapman et al., 1998; Koren and Bentires-Alj, 2015; Sun and Yu, 2015). Two proposed models eloquently explain heterogeneity in cancer: the stochastic model and the cancer stem cell model (Figure 20.1/Plate 19). The stochastic model of cancer development argues that all cells of a tumor have the intrinsic potential to divide and to support tumor growth. However, the cells are not organized in a sequential system and can choose, at random, between self-renewal or differentiation (Sidow and Spies, 2015). The cancer stem cell (CSC) model proposes that tumors demonstrate a hierarchical manner, as do normal tissues of the body regarding growth, with the CSC as the initiating point of tumor growth (Gonzalez-Moles et al., 2013; Lang et al., 2015; Maenhaut et al., 2010; Plaks et al., 2015). These cells can self-renew extensively and can also differentiate to produce specialized tumor mass (Shackleton et al., 2009). As of today, the CSC hypothesis suggests the presence of specific tumor cell populations responsible for distinct clinical scenarios, including but not

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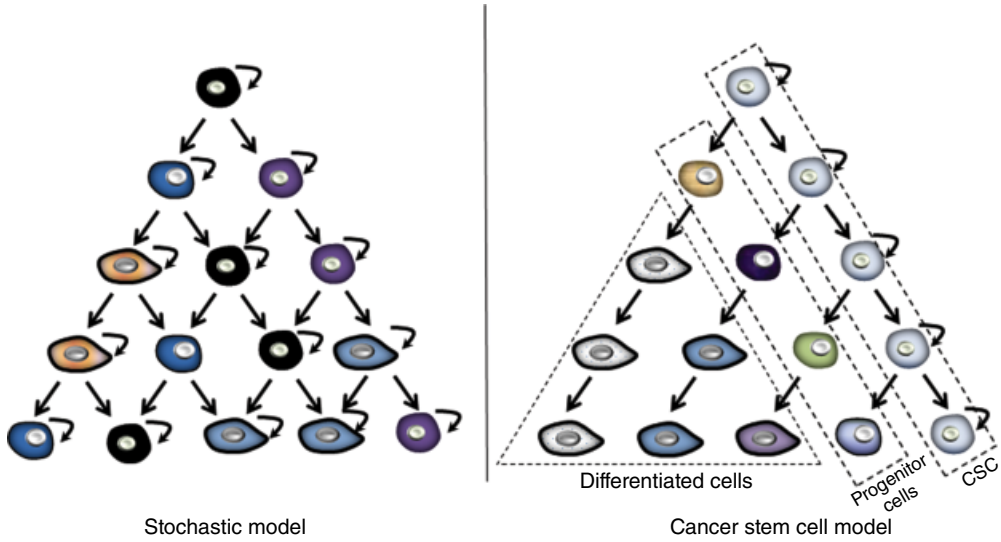

Figure 20.1 (Plate 19) Proposed models of cancer development and heterogeneity. *The stochastic model of cancer development advocates that all tumor cells have similar potential to divide and support growth of the tumor. These tumor cells are equipotent and can choose stochastically between self-renewal and differentiation. The cancer stem cell (CSCs) model of tumorigenesis proposes a hierarchical manner of cancer development and growth. According to this model, CSCs are the only cells with the potential to proliferate extensively and generate committed progenitor cells. These multiple progenitor cells can then further give rise to more differentiated cells, thus augmenting heterogeneity of the tumor mass. (See insert for color representation of the figure.)*

limited to initial tumor formation, relapse after initial therapy, progression from benign to metastatic disease of solid tumors, and transformation to a highly aggressive state in hematological malignancies (Economopoulou et al., 2012; Lawson et al., 2009; Shackleton et al., 2009). Despite significant evidence, the role of CSCs in cancer remains a popular area of active research and of intense debate. In this chapter, we briefly review the concept of stem cells, CSCs, the methods of identification and isolation of CSCs and their significance in disease development and progression. Moreover, we provide in-depth discussion about the genes and signaling pathways regulating CSCs and the potential therapeutic strategies targeting CSCs for anticancer treatments.

20.2 Stem Cells and Cancer: Historical Perspective

Stem cells are a ubiquitous constituent of nearly all multicellular organisms. The fundamental characteristic of stem cells is their ability to renew in order to maintain their stable population, and to differentiate into a wide range of cells of specialized functions (Spradling et al., 2001). In humans, such cells are present either transiently; during embryonic development, that is embryonic stem cells (ESCs); or throughout the life of the individual, adult stem cells (ASCs). ESCs are present in the inner mass of the blastocyst and are pluripotent in nature. They have the ability to give rise to any type of adult cell and can be maintained in culture indefinitely in their undifferentiated state. Due to their resilient nature, much hope has been placed in ESCs providing multiple therapeutic applications. However, the possibility of teratoma formation, ethical restrictions, and so on has limited the use of ESCs in regenerative medicine.

With the subsequent growth of the embryo, ESCs mature into multipotent stem cells, also called progenitor cells. These differentiated stem cells are committed to generate specific cell types with distinct functions: for example, the stem cells of the neural system that give rise to the three main types of cells present in adult brain, and the stem cells of the hematopoietic system that give rise to cells of the blood (i.e., platelets, erythrocytes and white blood cells) (Murry and Keller, 2008). Thus, the multipotent stem cells divide to generate a progeny of specialized cells, which are the unipotent stem cells. These unipotent stem cells, as the name implies, are capable of dividing into only one lineage of cells that are the nullipotent cells and constitute much of the organ. The multipotent stem cells represent the ASCs and differentiate into specific lineages depending upon the organ and the cell of origin. Theoretically, ASCs are considered to give rise to a limited cell type; however, recent evidence has emerged that suggests a significant degree of cellular plasticity in these ASCs. Moreover, studies have shown that ASCs, under the influence of the microenvironment can be forced to generate cell types other than their tissue of origin, suggesting that ASCs may alter their cell fate (Dulak et al., 2015). Although this observation has faced considerable criticism, the ability of stem cells to change fate holds immense therapeutic potential (Girlovanu et al., 2015). However, ambiguity in the concept of stem cell plasticity still exists and rigorous experimental proof is required to successfully validate this concept.

While a renaissance in CSC research has been observed in recent years, the concept of stem cells and cancer is several decades old with the earliest reports on CSCs put forth in 1800s. Rudolf Virchow, in 1858, postulated that cancers arise from embryo-like cells (Balkwill and Mantovani, 2001). Later in 1875, Julius Cohnheim elaborated Virchow's hypothesis, proposing that residual embryonic cells lie dormant through development, then, when elicited, become cancerous and lead to tumor development (Hamburger and Salmon, 1977). The "embryonal rest" theory by Virchow and Cohnheim postulates there are observable histological similarities between teratocarcinomas and the developing fetus (Balkwill and Mantovani, 2001). While these hypotheses attempt to explain teratocarcinomas, cancers primarily observed in children, they do not elaborate on the origin of acquired cancers arising in adulthood.

About a century later, Till, McCulloch and colleagues made the vital observation that cells from the bone marrow could generate myeloerythroid colonies in the spleen, when cultured in irradiated mice. When these experiments were extended to colony-forming assays in culture conditions with myeloblastic leukemia, they observed self-renewal capacity and abnormal patterns of differentiation of these cancer cells (Becker et al., 1963; McCulloch and Till, 1964; Siminovitch et al., 1963; Till and McCulloch, 1961, 1980; Worton et al., 1969). Methods were quickly adapted for the assay of clonogenicity of other hematological malignancies (Metcalf et al., 1988; Metcalf, 1982; Williams et al., 1988) and solid tissue ovarian cancers (Buick and Fry, 1980; Buick et al., 1985). In relatively recent years, Weissman and co-workers (Spangrude et al., 1988) rekindled the interest and relationship between cancer and tissue stem cells when they isolated multipotential hematopoetic progenitor cells and their progeny, using multiparameter FACS, with the aid of monoclonal antibodies. Subsequently, technology was also expanded in identifying hematopoietic stem cells (HSCs) counterparts in leukemia with established *in vitro* clonogenic assays. In a series of elaborate experiments, Dick and colleagues identified and isolated leukemia stem cells (LSCs) and further demonstrated that these cells were capable of repopulating tumors in non-obese diabetic severe combined immunodeficient (NOD/SCID) mice, thus, establishing the foundation of the CSC hypothesis widely accepted today.

20.3 Cancer Stem Cells

The development of an organ requires careful orchestration of signaling pathways in multipotent stem cells and leads to the gradual maturity of these cells into a heterogenous population constituting a functional organ. During the development of a tumor, the pathways regulating self-renewal and differentiation are aberrantly modulated, promoting uncontrolled and enhanced proliferation (Studzinski, 1989). The highly recognized

role of ESCs in embryogenesis, and of ASCs in maintenance of adult tissues (e.g., blood, liver, etc.), stimulated the idea of a parallel model in cancer: subsequently, an inundation of literature on stem cells in cancer followed (Shay, 1997; Tan et al., 2006). These cells were later termed as cancer stem cells (CSCs), tumor initiating cells (TICs), tumor-maintaining cells (TMCs), and so on. While all these terms are directed to the same population of cells, TMCs or tumor-propagating cells (TPCs), we believe, would be the most appropriate expression to identify this subpopulation. While the concept of CSCs and implications are currently under intense investigation, three general properties have been put forth to define CSCs, and are listed in the order proposed: (1) expression of specific repertoire of cell surface markers, (2) demonstration of tumorigenic capacity as compared to other subset of cells, and (3) capacity to establish the complete gamut of cancer cells, recapitulating the heterogeneity of the parent tumor.

Furthermore, due to the common points between the CSCs and ASCs, other characteristics explicitly associated with these somatic stem cells (SSCs) were also suggested for CSCs (i.e., CSCs associated niche, asymmetric division of CSCs, irreversible transition of the CSCs to derived cells, resistance to hypoxia, etc.). Clinical relevance of the CSC concept categorizes CSCs as a chemotherapeutic- and radiotherapy-resistant subpopulation of cells, thus, the potential subpopulation responsible for the recurrence of cancer after therapies (Ailles et al., 1997). Therefore, the CSC concept proposes that a small subpopulation of cells within tumor cells possess cancer-initiating properties; this subset is able to maintain tumor growth by sustaining their ability to generate progeny of both self-tumorigenic cells and a differentiated non-tumorigenic tumor bulk population. This concept implies that the majority of the tumor cells lack self-renewal ability and cannot recapitulate tumor growth upon xenotransplantation. Moreover, the requirement of a large number of regular tumor cells to develop a cancer in an immunodeficient mouse also adds to the CSC hypothesis; however, another possible explanation would be that certain tumor cells have greater potentials to proliferate and differentiate, building the different types of tumor cells.

20.3.1 The Origin of Cancer Stem Cells

While researchers have made significant strides in experimental approaches to study CSCs; their origin, maintenance and requirement in tumor progression has been largely disputed. In recent years, the requirement for more promising and effective therapies has fueled the requirement of CSC research along with the emergence of several hypotheses to explain the origin and maintenance of CSCs (Figure 20.2/Plate 20).

The first evidence on the existence of CSCs was demonstrated by successful initiation and development of tumor in NOD/SCID mice with the transplantation of primary acute myeloid leukemia (AML) (Bonnet and Dick, 1997). Further, using a series of transplantation experiments, it was demonstrated that the leukemia-initiating cells expressing a $CD34^+/CD38^-$ phenotype had high self-renewal capacity; in contrast, it was observed that $CD34^+/CD38^+$ phenotypes were not able to initiate leukemia in immunodeficient mice. Therefore, the cells with $CD34^+/CD38^-$ phenotype were acknowledged as AML stem cells, that is, leukemia stem cells (LSCs) (Al-Hajj et al., 2003). This notion further led to the identification of stem cells in solid tumors. Subsequently, it was observed that while millions of cells are required to establish a tumor in xenograft mice, as little as a few hundred of these putative CSCs can produce a tumor in similar experiments. While substantial information has been generated verifying the presence of CSCs in cancer, the origin of stem cells still remains controversial and several hypotheses have been described to understand the origin of CSCs and tumor heterogeneity.

20.3.1.1 Genetic Instability and Cell Fusion

Genetic instability of normal cells has been identified as the fundamental basis for cell transformation and subsequent cancer initiation. Modifications resulting in instability may occur at chromosomal or molecular levels. Most frequent alterations at the genomic levels involve the gain or loss of chromosome or

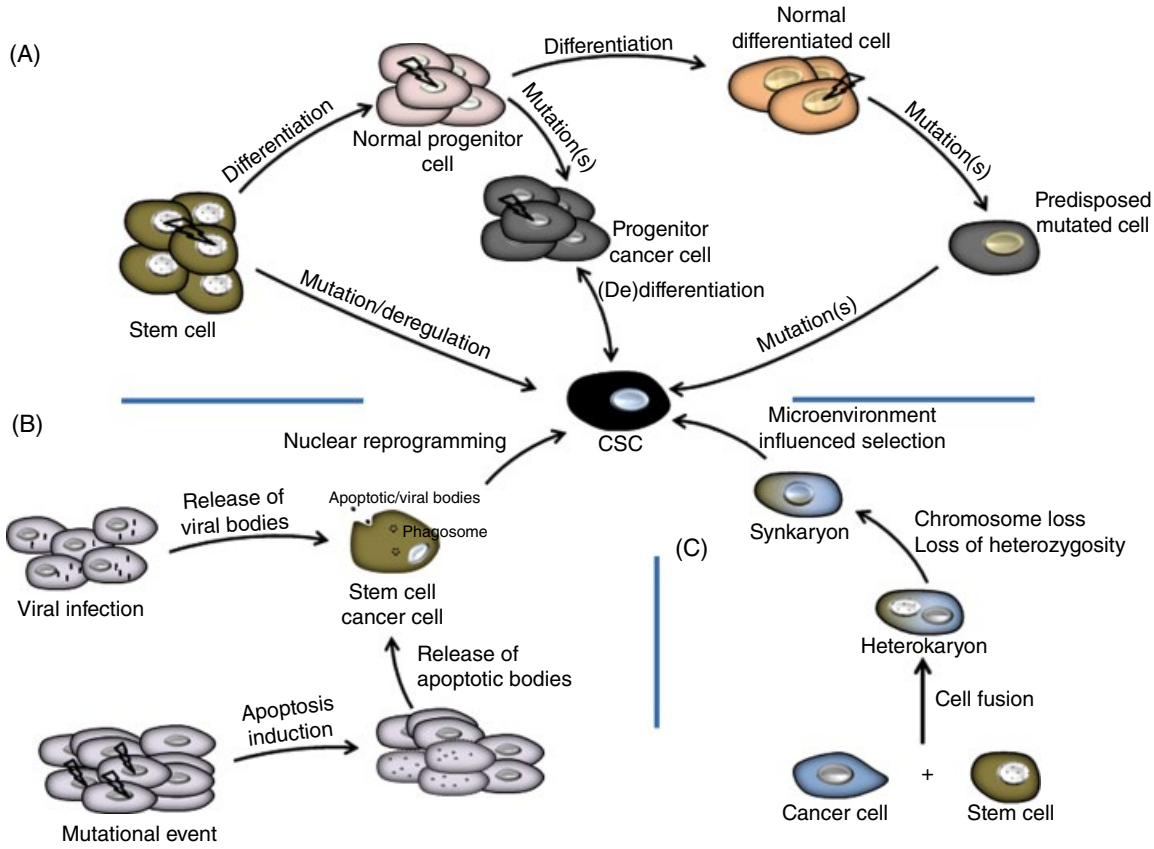

Figure 20.2 (Plate 20) Origin of cancer stem cells. *The presence of cancer stem cells (CSCs) has been verified in several tumors, and various hypotheses have been proposed to understand the formation of CSCs: (A) CSCs can arise as a result of mutation and/or generic abnormalities in a normal stem cell, or from the progenitor cancer cell generated upon mutations in normal progenitor cells which re-acquire self-renewal ability, and/or of from a normal cell which may acquire mutations predisposing it to form potential CSCs after further mutations; (B) apoptosis of somatic cells in response to stress may cause release of fragmented DNA. These fragmented DNA can be taken up by other stem/progenitor cells through endocytosis or phagocytosis causing nuclear reprogramming of the acceptor cell and formation of potential CSCs. Furthermore, due the presence of viral particles, viral oncogenes can also be taken up by stem cells potentially reprogramming it to generate CSCs; and (C) fusion of cancer cells with a normal stem cell can lead to the generation of heterokaryon (multinucleated cell) or synkaryon (mononucleated cell). Loss of heterozygosity in heterokaryons leads to the generation of synkaryons. These hybrid cell thus generated may possess self-renewal activity as well as properties of transformed cells, that is, properties of CSCs. (See insert for color representation of the figure.)*

chromosomal derangements generating so-called aneuploidy (Sen, 2000). These alterations can lead to significant genomic instability, potentially causing an imbalance in chromosome number and a loss of heterozygosity. Moreover, at the molecular level, point mutations may augment activation of oncogenes or suppression of tumor suppressor genes, enhancing susceptibility to tumorigenesis (Hansen and Cavenee, 1988; Ilyas et al., 1999). While more studies are required to identify whether aneuploidy is an effect or cause of tumorigenesis, it has been observed that carcinogens such as arsenic and asbestos generate aneuploidy, before the development of frank malignancy, suggesting their role in cancer-initiation.

While mutations are eliminated from the pool of cells through extensive defense mechanisms of DNA repair, and in extreme cases, through elimination by apoptosis, some mutations may accumulate at low frequencies that can be accelerated by toxicants or carcinogens. Certain genetic or epigenetic modifications in cells lead to increased proliferation causing enhanced accumulation of genetic mutations. These alterations can account for selective advantage of clonal expansion in the mutated cells depending on the tumor microenvironment, cell-substratum, and cell-cell interaction; furthermore, stem cells are conferred an additional advantage in this situation due to their relatively long-life and slow proliferation. Thus, it can be concluded that both mutations and chromosomal aberrations work in tandem in the stages leading to cancer development and both of these events can be responsible for the generation of CSCs.

Another physiological event capable of introducing genomic instability in normal cells or stem cells is cell fusion. Numerous biological functions and cellular developments such as fertilization, formation of tissues (placenta, bone, and muscle), tissue regeneration and repair, immune response, etc. require cell-cell fusion (Alvarez-Dolado et al., 2003; O'Malley, 2004; Pomerantz and Grandis, 2004; Weimann et al., 2003). Circulating, normal hematopoietic stem cells have been observed to fuse with different types of cells such as cardiomyocytes and hepatocytes; therefore, the development and progression of cancer can result from cell fusion and hybridization between leukocytes and somatic cells. Further, significant evidence demonstrates that fusion of lymphocytes with tumor cells leads to the formation of metastatic cells (Mekler, 1971); moreover, this fusion has also been indicated to enhance the genotypic and phenotypic heterogeneity of tumors (Larizza et al., 1984; Rachkovsky et al., 1998). The fusion of two cells results in the formation of either multinucleated (heterokaryon) or mononucleated (synkaryon) cells. Viral-mediated fusion of murine Ehrlich ascitic cells with HeLa cells was the first observed heterokaryon *in vitro* (Harris, 1965). These heterokaryons are observed over a period of time possessing demonstrable characteristics and functions of each partner. Furthermore, the formation of synkaryons requires the intermediary generation of heterokaryons and features chromosomal loss after nuclear fusion. Chromosomal loss during the cell-cell fusion of a transformed cell with a normal stem cell may lead to the formation of CSCs by incorporating cancer-promoting properties of the transformed cells along with cell survival ability of the stem cell (characteristics of CSCs), thus challenging the concept of transdifferentiation due to the somatic stem cell plasticity.

Several fusogenic factors that are species-specific and cell-type specific have been recognized. CD44, CD47, and PTPNS1 (macrophage fusion receptor) have been identified as potential fusogenic proteins in mammalian cells (Vignery, 2000). Several cell fusion regulating proteins have been recognized in *C. elegans*, and their role in organ development and mechanism of fusion of cells has been extensively studied (Shemer and Podbilewicz, 2003). Several human homologs of the *C. elegans* proteins have also been identified, for instance, HOXA5 (homeobox A5) is the human homolog of *C. elegans* lin-39 fusogenic factor (Bel-Vialar et al., 2000; van der Lugt et al., 1996). Interestingly, Wnt, Notch, and Hedgehog signaling pathways, regulating cancer progression and development, demonstrate cross-regulatory interactions with various homeobox genes (Basu and Roy, 2013; Bondos, 2006; Daikoku et al., 2004; Moreno, 2010). Furthermore, several reports indicate the fusion of tumor cells with various types of cells present in the tumor microenvironment. Mesenchymal stem cells have been identified as an important fusogenic candidate for the propagation of tumors as well as generation of genetic diversity leading to formation of reprogrammed cells with slow-growing and stem-cell like characteristics (Wei et al., 2014). Transcriptomic profiling of fused cells identified FOXF1 as the mediator of stem-like reprogramming (Wei et al., 2014). Thus, the cell fusion process may contribute to the development of CSCs and initiation of cancer.

In addition to fusogenic factors, certain cytokines and chemokines have also been implicated in facilitating or increasing cell fusion processes with their specific roles identified in the fusion of normal cells. For example, the chemokine SDF1 (also known as CXCL12) and its receptor CXCR4, known to have an important function in the metastasis and progression of cancer as well as the trafficking of normal stem cells and CSCs, have recently been demonstrated to promote monocyte fusion *in vitro* (Shemer and Podbilewicz, 2003).

Furthermore, the cytokine interleukin-4, functioning through its receptor, has been observed *in vitro*, to facilitate fusion of myoblasts with myotubes, a critical step of muscle development. Meanwhile, CXCR4 is highly expressed in a wide variety of cancers such as pancreatic, prostate, and hematological malignancies. Similarly, the IL-4 receptor has been identified as a crucial regulator of different types of cancers such as brain, lung, and breast. These findings imply, although indirectly, that the activation of chemokine and cytokine signaling axes in cancer cells might enhance the incidence of cell-cell fusion events.

20.3.1.2 Horizontal Gene Transfer

Bacteria and fungi have long been known to institute resistance against antibiotics through horizontal gene transfer. After an apoptotic cell death, the generated apoptotic bodies are engulfed by neighboring cells through phagocytosis. Transfer of DNA can result from phagocytosis or endocytosis of apoptotic cells, microvesicles, or cell-free DNA, by recipient cells, through the mechanisms of transduction, transfection or conjugation. Horizontal transfer of genes is essentially a three step process: *one*, the delivery of donor DNA to recipient cells; *two*, uptake and insertion of the sequences into the genome; and *three*, the expression of the incorporated genes for the benefit of the beneficiary cell (Bergsmedh et al., 2001). However, contrary to the accepted belief that DNA is degraded and rendered inactivated in cells undergoing apoptosis, thus making it harmless to recipient cells, it has been observed that the leftover DNA in apoptotic cells is transferred to recipient cells in a relatively intact form. Therefore, it is speculated that the apoptotic bodies of cells with genetic mutations capable of cancer initiation and progression, can induce nuclear reprogramming of recipient cells followed by their transformation into tumorigenic cells (Holmgren et al., 1999). Horizontal transfer of genes was first observed in cells treated with conditioned media of Epstein-Barr virus (EBV); integrated cells were found to express EBV encoded genes at both the mRNA and protein levels (Holmgren et al., 1999). Moreover, apoptotic bodies from tumor cells have been observed inducing p53-deficient fibroblasts to form colonies *in vitro* and *in vivo*. Apart from endocytosis, the formation of a complex network of tunneling nanotubes, for selective transfer of membrane vesicles and organelles between cells, has also been observed (Rustom et al., 2004), identifying a potential active method of transfer of molecules between cells. More recently, it was demonstrated that cell-free DNA from the serum of colon cancer patients and conditioned media of SW480 human colon cancer cells can induce transformation and tumorigenesis in NIH3T3 murine cells (Trejo-Becerril et al., 2012). Thus, horizontal gene transfer through phagocytosis or transduction may have a role in the initiation of a tumor and the generation of CSCs.

20.3.1.3 Microenvironment

While genomic abnormalities resulting from mutations and chromosome derangements may trigger CSC formation, the microenvironment of the transformed cells influences selective clonal expansion and dedifferentiation of CSCs in a cancer tissue. It is now known that numerous factors in the microenvironment, under specific conditions, (such as infection or injury), regulate stem cell differentiation and initiate tumorigenesis. For example, the inflammatory microenvironment has been attributed to initiation and progression of a number of tumors such as breast, prostate, pancreas, liver, and so on. The production of inflammatory cytokines and chemokines and other DNA damaging agents, participates in CSC formation and regulates their equilibrium with less tumorigenic non-CSCs subpopulation. It has been observed in conversion of non-CSCs to CSCs under the influence of IL-6, which begins with the dedifferentiation of non-CSCs (Figure 20.3/Plate 21). Furthermore, the activation of NF-κB, STAT, notch, and hedgehog (etc.), signaling pathways contributes to the development and maintenance of CSCs. Tumor infiltration of immune cells, and differentiation of stromal fibroblast cells, also enhances the concentration of cell-free signaling molecules and other components, eliciting CSC formation. Thus, understanding the role of the microenvironment

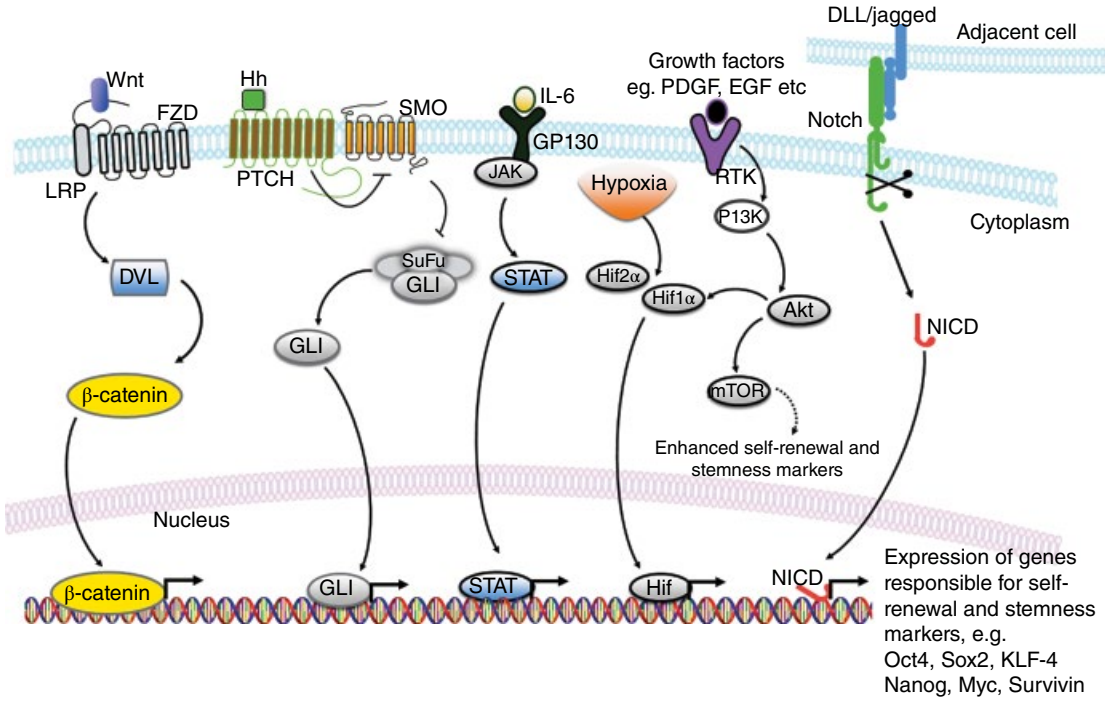

Figure 20.3 (Plate 21) Signaling pathways frequently utilized by cancer stem cells. *Transcription factors downstream of the represented signaling pathways have been associated in generation and maintenance of CSCs in different cancer types by upregulation of transcription factors such as Oct3/4, Sox2, Nanog, KLF-4, and so on, and stemness associated genes. These transcription factors further reinforce stemness and stem cell markers enhancing tumorigenecity and maintenance of CSC sub-population. DVL, Dishevelled homolog; EGF, Epidermal growth factor; FZD, Frizzed; GP130, membrane glycoprotein 130; Hh, Hedgehog; HIF, Hypoxia inducible factor; IL-6, interleukin-6; JAK, Janus kinase; mTOR, mammalian target of rapamycin; NICD, Notch intracellular domain; PDGF, platelet-derived growth factor; PI3K, Phosphoinositide 3-kinase; PTCH, protein Patched homolog; RTK, receptor tyrosine kinase; SMO, Smoothened; STAT, signal transducer and activator of transcription; SUFU, Suppressor of fused homolog; Wnt, Wingless-type. (See insert for color representation of the figure.)*

will help to develop more effective cancer interventional therapies. In a seminal study exploring differentiation potential and the plasticity of tissue-specific stem cells, Galli et al. (2000) demonstrated that when neural stem cells isolated from mice were cultured with myoblasts, these stem cells differentiated into muscle cells. Thus, these observations indicate the influence of a tissue-specific microenvironment on the differentiation of stem cells.

20.4 Identification and Isolation of CSCs

CSCs were first identified by John Dick's group in 1994, by sorting the cells based on cell surface biomarker ($CD34^{+}CD38^{-}$) in acute myeloid leukemia and were also confirmed by *de novo* reforming in immunodeficient mice upon transplantation (Lapidot et al., 1994). Since the discovery of highly tumorigenic CSC subpopulation in cancer, methods to accurately identify and isolate CSCs has been explored, in an effort to study their biological characteristics. Presence of CSCs has been analyzed in primary patient tumor samples, mice

tumors, human cancer cell lines, and mice xenograft models. The identification of CSCs in stable cell lines is relatively easy; unfortunately, it cannot recapitulate the entire primary tumor used to generate these cell lines. While patient samples for the identification of CSCs is the gold standard, the availability of fresh tumors hampers large-scale studies (Keysar and Jimeno, 2010).

It still remains unclear why only a small population of tumor cells are more tumorigenic than others and what molecular mechanisms govern the characteristics and differentiation of CSCs. Presence of CSCs is now confirmed in different kinds of cancers, including leukemia, glioblastoma, brain tumors, multiple myeloma, gastric cancer, pancreatic cancer, colon cancer, and so on. However, the precise identification and analysis of the subpopulation of CSCs and development of chemotherapeutic agents with CSC-inhibiting activity is still under intense investigation. To date, the most common technique for the isolation of CSCs employ flow cytometry and cell sorting approach, utilizing specific phenotypic fingerprints and confirmation of their biological activity through serial implantation in mice. Broadly the CSC markers are identified by presence of unique cell surface markers, such as proteins of cluster of differentiation (CD) and intracellular markers, such as ALDH activity. It has been postulated that patients with enhanced levels of CSCs in their tumors will demonstrate an aggressive form of the disease, expressing significant resistance to current treatment modalities. Expression of phenotypic cell surface markers is a hallmark for isolation of CSCs, and its biological validation separates them from normal cancer cells. Phenotypic surface markers have been used to isolate CSCs in cancer present in different parts of the body or in different cell lines. Multiple cell surface markers expressed individually or in combination, increase stringency of CSC separation from other cancer cells. Moreover, differential expression and distribution pattern of CSC markers in cancers from variable histological grades and degrees of distribution has been observed. Thus, it is necessary to identify the biomarkers of CSCs in tumors and devise effective methodology for studying their biological properties. Some of the markers presently used to identify CSCs are discussed in the following.

20.4.1 CD133

CD133, a member of pentaspan transmembrane proteins, specifically localizes in cellular protrusions and has also been used as candidate for the discrimination of CSCs. CD133 is one of the most studied and extensively used surface markers to separate regenerating cells from normal cells. It has been identified as a CSC surface marker in solid tissue such as glioblastomas and medulloblastomas, epithelial tumors such as ovarian, liver, pancreas gastric, cutaneous melanomas, and colon cancers. The $CD133^+$ cells are validated as the CSCs subpopulation when O'Brien et al. demonstrated that subcutaneous injection of $CD133^+$ colon cancer cells were capable of recapitulating tumor heterogeneity similar to the original tumor in NOD/SCID mice. Furthermore, $CD133^-$ colon cancer cells were not able to form a tumor, thus, confirming the stemness of CD133 subpopulation (Ricci-Vitiani et al., 2007). Cells expressing stem cell marker CD133 show plasticity with enhanced colony formation and cell motility in colon and osteosarcoma tumors (Elsaba et al., 2010; Tirino et al., 2008). $CD133^+$ cells are also found to contribute cancer tumorigenecity, pathology, and recurrences in endometrial, metastatic colon, ovarian, gallbladder, and hepatocellular carcinoma (Friel et al., 2010; Kryczek et al., 2012; Shi et al., 2010; Shmelkov et al., 2008; Zhu et al., 2010). The precise mechanisms regulating CD133 expression and its functions in CSCs remain unknown. However, there is increasing evidence indicating that CD133 is also a CSC-specific marker in combination with other surface molecules such as $CD44^+$/$CD24^+$/$CD133^+$ in melanoma (Dou et al., 2007), $CD44^+$/$CD133^+$ in prostate cancer (Oktem et al., 2014), $CD133^+$/$CD19^-$/$CD38^-$ in acute lymphoblastic leukemia (Cox et al., 2009), $CD133^+$/Bmi-1/ABCG2 in ameloblastic tumors (Kumamoto and Ohki, 2010), $CD133^+$/$CD44^+$ in hepatocellular carcinomas (Salnikov et al., 2009), and so on. Moreover, CD133 marker expression functions as a predictive marker of metastasis and survival after chemotherapy of rectal cancer. Thus, $CD133^+$ cells have potential in investigating the tumorigenic process involving CSCs.

20.4.2 CD24

CD24, a small heat-stable, highly-glycosylated cell surface molecule, functions in cell adhesion as it is linked to the membrane through a glycosylphosphatidylinositol anchor. Expression of CD24 in normal individuals is limited to B-cells, the stratum corneum of the epidermis and the granulocytes. Thus, the expression of CD24 markers on the cell within a tumor is a characteristic feature for the unique identification of CSCs. Presence of CD24 surface markers are observed in many human carcinomas and its expression is linked to poor prognosis, tumor invasion, and migration. Moreover, CD24 has been demonstrated to be a downstream molecule in the hedgehog developmental pathway often significantly upregulated in cancers and CSCs. $CD24^+$ cells isolated from clinical ovarian tissues samples reveal an enriched population of stem-cell like cells. *In vitro* experiments show that cells with $CD24^+$ phenotype display an enhanced capacity for self-renewal and chemoresistance as compared to $CD24^-$ cells. Likewise, $CD24^+$ cells express higher levels of stemness-associated genes, such as Bmi-1, Oct3/4, Notch1, and Notch4. Moreover, implantation of only 5000 $CD24^+$ cells is able to form tumors in nude mice, whereas an equal number of $CD24^-$ fails to induce tumor growth. Similarly, side population (SP) cells of pancreatic cancer cells are enriched with $CD24^+$/$CD44^+$/$CD133^+$ as compared to non-SP cells and also SP cells demonstrate increased tumorigenecity upon xenografting in nude mice and enhanced chemoresistance against gemcitabine. $CD24^+$ is recognized as a CSC marker in combination with other biomarkers such as, $CD44^+$/$CD24^+$ in colorectal cancer (Yeung et al., 2010), $CD133^+$/$CD44^+$/$CD24^+$ in murine melanoma (Dou et al., 2007), $CD44^+$/$CD24^+$/$CD326^+$ in pancreatic cancer (Li et al., 2007).

However, conflicting data reflecting lack or low expression of CD24 has also been used to identify CSCs, thereby reducing the unanimous usefulness of this marker in identification of CSCs in all tumors. $CD44^+$/$CD24^{-/low}$ phenotype expressing cells were first characterized as breast CSCs (Al-Hajj et al., 2003). The basal subtype of breast cancer cells of patient tissues and cancer cell lines exhibiting $CD44^+$ /$CD24^{-/low}$ phenotypes encompass an enriched population of cells undergoing EMT. In addition, it has been observed that $CD44^+$ prostate CSCs migrated through Matrigel and suppressed CD24 expression. Thus, these findings suggest that either the presence or absence of CD24 may be used as a putative marker for CSCs. However, the role of CD24 in all tumor types should be further analyzed.

20.4.3 CD44

CD44, a cell surface glycoprotein and receptor for hyaluronan (HA), is also involved in a number of physiological activities of cells associated with pathological signaling of cancer cells. CD44 is a multifunctional cell surface marker involved in cell adhesion, angiogenesis, cell migration, and the presentation of chemokine, cytokines, and growth factors to the analogous ligands. It has been demonstrated that when CSCs undergo epithelial-to-mesenchymal (EMT) transition they upregulate the expression of standard CD44. CD44 was first reported as an identification marker for head and neck carcinoma (Ailles and Prince, 2009), then later it was also detected in gastric cancer, prostate cancer (Wang et al., 2012), and glioblastoma (Takaishi et al., 2009). $CD44^+$ cells from gastric tumors are observed to show resistance to chemotherapy. CSC tumorigenesis involving $CD44^+$ is enhanced by the activity of Wnt and prostaglandin E2. Co-expression of CD44 with other markers has also been observed in many types of cancers such as breast - $CD44^+$/$CD24^{-/low}$ (Al-Hajj et al., 2003), colon - $CD44^+$/$CD133^+$ (Jing et al., 2015), prostate - $CD44^+$/$CD133^+$ (Oktem et al., 2014), pancreatic - $CD44^+$/$CD24^+$/$EpCAM^+$ (Li et al., 2007), murine melanoma - $CD133^+$/$CD44^+$/$CD24^+$ (Dou et al., 2007), HCC - $CD133^+$/$CD44^+$ (Kumamoto and Ohki, 2010), and ovarian cancer - $CD44^+$/$CD117^+$, or $CD133^+$/$CD117^+$ (Zhang et al., 2008). It was demonstrated that when 100 dissociated cells of spheres, from non-adherent ovarian cancer cells cultured under sphere forming conditions, were injected into female athymic BALB/c nude mice, full recapitulation of the original tumor

was generated, whereas >10^5 cells, grown in standard culture conditions, remained nontumorigenic. These sphere-forming cells were immunostained for surface phenotypes; significant upregulation of the surface receptors of CD44 and CD117 was observed (REF). Similarly, the inoculation of only 100 ovarian cells with $CD44^+/CD117^+$ phenotype could be serially propagated; while 10^5 CD44-/CD117- ovarian cells were not tumorigenic in athymic BALB/c nude mice. Thus, the presence of cells with $CD44^+/CD117^+$ phenotype represents a subpopulation of ovarian cancer responsible for development, progression and metastasis of ovarian cancer (Liu et al., 2010). The highly invasive prostate cancer cells with $CD44^+$ phenotype also had enhanced expression of BMI1, Nanog, and SHH with a genetic signature similar to stem cells (Klarmann et al., 2009). In most forms of prostate cancer, CD44 and CD133 double positive cells are reported to have the self-regenerating properties. In prostate, gall bladder, colon, liver, ovarian, and pancreatic cancer, CSCs have been isolated and separated on the basis of double positive cells for CD133 and CD44 (Collins et al., 2005; Haraguchi et al., 2006; Kryczek et al., 2012; Li et al., 2007; Shi et al., 2010; Zhu et al., 2010). In addition, high surface expression of integrin $\alpha 2\beta 1$ is utilized for the isolation of CSCs in prostate cancer (Collins et al., 2005).

20.4.4 EpCAM

Epithelial cell adhesion molecule (EpCAM/CD326) was first identified as the dominant antigen in human colon carcinoma (HCC) (Herlyn et al., 1979). It is a homophilic, $Ca2^+$-independent adhesion membrane glycoprotein. The protein structure comprises an epidermal growth factor (EGF) - and thyroglobulin repeat-like extracellular domain, a transmembrane domain, and a short intracellular domain. A variety of human epithelial tissues, stem cells, progenitor cells as well as cancer cells express EpCAM, thus it is one of the markers, which identifies highly tumorigenic tumor cells. It is demonstrated that the growth and invasiveness of HCC may be predicted by the presence of $EpCAM^+$ cells in the tumor (Munz et al., 2005). Subpopulations of $EpCAM^+$ cells from HCC tumor specimens and cancer cell lines are identified and sorted using flow cytometry. This enriched subpopulation of cells demonstrates greater colony formation than their $EpCAM^-$ counterparts and displays hepatic stem cell-like traits. In similar transplantation assays, as few as a 100 $EpCAM^+$ cells are capable of initiating tumor formation in mice. Moreover, Munz et al. (2005) also observe that $EpCAM^+$ cells can differentiate and form clones of both $EpCAM^+$ and $EpCAM^-$ both *in vitro* and *in vivo*; however, the $EpCAM^-$ subpopulation of cells sustain their phenotype. These studies identify HCC cells with $EpCAM^+$ as cells with preferentially high tumorigenic potential and CSC characteristics. Furthermore, in several tumors EpCAM has been demonstrated as a CSC specific marker in combination with other markers such as breast cancer ($EpCAM^+/CD44^+/CD24^{-/low}$) (Al-Hajj and Clarke, 2004), colorectal cancer ($EpCAM^{hi}/CD44^+$) (Liu et al., 2014), and pancreatic cancer ($CD44^+/CD24^+/EpCAM^+$) (Munz et al., 2005).

20.4.5 CD177

CD177, also known as c-kit, is a proto-oncogene and a stem cell growth factor receptor also used to identify stages of differentiation and activities of hematopoietic stem cells in bone marrow. It has been demonstrated that when tyrosine kinase binds to the receptor, intrinsic kinase activity of CD177 leads to the activation of cell survival, and proliferation signaling. Presence of CD177 in combination with $CD44^+$ on cells identifies ovarian cancer cells with enhanced growth rate in cell culture and after injection into nude mice House (Chen et al., 2013). Moreover, osteo-carcinoma cells with Stro-1^+ and $CD117^+$ phenotypes have shown self-renewability, multi-potency, and drug resistance, properties associated with CSCs. It was observed that Stro-1^+ $CD117^+$ cells efficiently initiate cancer in mice, whereas their counterpart Stro-1^-CD117^- cells fail to initiate tumor formation (Sharma et al., 2011).

20.4.6 CD34

CD34, a cell surface glycoprotein, functions as a cell-cell adhesion factor for T-cells and lymph nodes; it assists in the attachment of a stem cell to a stromal cell. $CD34^+$ sub-population of cells shows stem cell properties in hematopoietic malignancies while negative CD34 cells lack the same characteristics (Kuranda et al., 2011). $CD34^+$ and $CD38^-$ group of cells produced human acute myelogenous leukemia in severe combined immunodeficient and nonobese diabetic mice, whereas $CD34^+$ $CD38^+$ and $CD34^-$ cells were not able to produce the same disease (Lapidot et al., 1994). The same pair of markers ($CD34^+$ $CD38^-$) has been used to isolate and characterize disease in human acute mylogenous leukemia and acute lymphoblastic leukemia (Bonnet and Dick, 1997; Castor et al., 2005).

20.4.7 ALDH1

Aldehyde dehydrogenases (ALDH) belong to the oxidoreductase family, catalyzing the conversion of aldehydes to their corresponding acids. These enzymes are mainly involved in cellular detoxification, differentiation and drug resistance, through the oxidation of cellular aldehydes (Moreb et al., 1996). ALDH1 oxidizes retinol to retinoic acid, which is a modulator of cell proliferation, thus it may have a role in early differentiation and proliferation of SCs (Mieog et al., 2012). It has been demonstrated that ALDH positive melanoma cells are more tumorigenic than ALDH negative cells in nude mice experiments. *In vivo*, ALDH1 silenced by siRNA/shRNA leads to reduced tumorigenesis, moreover, *in vitro* inhibition of ALDH leads to apoptosis, decreased cell viability and cell cycle arrest. ALDH1-positive CSCs have been observed to mediate metastasis and poor clinical outcome in inflammatory breast cancer (IBC). Charafe-Jauffret et al. showed that CSCs isolated from SUM149 and MARY-X, an IBC cell line and primary xenograft, using ALDH1 activity, had enhanced invasive and metastatic potential assessed by *in vitro* and mouse xenograft assays (Charafe-Jauffret et al., 2010). Furthermore, ALDH1 expression in IBC or breast cancer was also identified as an independent predictive factor for early metastasis and decreased survival in this patient population. Thus, the metastatic, aggressive behavior of IBC may be mediated by a CSC component that displaying ALDH enzymatic activity (Ginestier et al., 2007, 2010). Su and colleagues investigated the stem cell-related function and clinical significance of the ALDH1A1 in bladder urothelial cell carcinoma. Stem cell or stem-like cancer cell characteristics of the $ALDH1A1^+$ cells were assessed by *in vitro* and *in vivo* approaches. They used immunohistochemistry assay to evaluate ALDH1A1 expression on 22 normal bladder tissues and on 216 bladder tumor specimens of different stages and grades. The results indicate that $ALDH1A1^+$ cancer cells display higher *in vitro* tumorigenecity and generate xenograft tumors that resemble the histopathologic characteristics and heterogeneity of the parental cells, whereas isogenic $ALDH1A1^-$ cells do not have similar effects. ALDH activity is now also utilized for the isolation of CSCs in nearly all forms of cancer like breast, ovarian, pancreatic, prostate, colon, and leukemia (Croker et al., 2009; Kryczek et al., 2012; Lugli et al., 2010; Ran et al., 2009; Rasheed et al., 2010; van den Hoogen et al., 2010).

20.4.8 CXCR4

The role of chemokines and their receptors in cancer progression and development has been studied extensively (Arora et al., 2013; Singh et al., 2004, 2012). Chemokine receptors are a family of GPCR-based transmembrane receptors classified into four groups of C, CC, CSC, and CX3C, based on position the of first two cysteine residues. The CXCR4 receptor is one of the best-studied chemokine receptor that binds selectively to its sole ligand CXCL12 (also known as stromal derived factor-1, SDF-1). CXCR4 expression is observed in a variety of normal cells. However, interestingly when cancer cells significantly overexpress CXCR4, little or no expression of CXCR4 is observed on adjacent normal tissue. This observation led to the suggestion that

malignant cells may be derivatives of CXCR4 expressing normal stem cells (Fredriksson et al., 2003). Further research indicates that CXCR4 is responsible for cancer metastasis and maintenance of CSCs (Muller et al., 2001). Later, Furusato et al. demonstrated that prostate CSCs express high levels of CD133, CD44 and CXCR4, providing further evidence for the role of CXCR4 in CSCs (Furusato et al., 2010). Furthermore, since the CXCL12-CXCR4 signaling axis is widely recognized as one of the master regulators of trafficking of both normal SCs and CSCs, and since CXCR4 is highly expressed on CSCs, it is postulated that the CXCL12-CXCR4 signaling axis also governs metastasis to organs (e.g., lungs, liver, lymph nodes, and bones), which highly express CXCL12. Thus targeting of this signaling axis may provide significant therapeutic application to inhibit the metastasis of tumor cells. Furthermore, the role of CXCR4 in CSC maintenance is observed in breast cancer cells, wherein exogenously added CXCL12 protects the breast cancer cells from apoptosis. Additionally, MDA-MB-231 cells expressing high levels of CXCR4 are observed to form large tumors in mice with high degree of metastasis to other organs in just 3 weeks; however, MCF7 cells of $CXCR4^{low}$ phenotype form small tumors in mice only after weeks of incubation and do not metastasize to other organs (Chauchereau et al., 2011). CXCR4 expressing CSC are identified in different types of cancers such as breast cancer (Hwang-Verslues et al., 2009), pancreatic cancer (Kucerova et al., 2010), melanoma (Hirbe et al., 2010), prostate cancer (Tomuleasa et al., 2010), acute myeloid leukemia (Mueller et al., 2010) and HCC (Dewan et al., 2006). However, further investigation on the role of CXCR4 in various CSCs remains to be explored.

20.4.9 Side Population

ATP-binding cassette (ABC) transporters and multidrug resistance transporter1 (MDR1) are membrane transporters that can pump small molecules of cytotoxic drugs and dyes out of the cells at the expense of ATP. This superfamily includes multidrug resistance proteins (MRPs/ABCC), breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (P-gp/ABCB1). The subpopulation of CSCs with high expression levels of ABC transporters are designated as side population (SP) and can be isolated by treatment of cells with Hoechst 33342. SP cells expel the dye and this subpopulation of CSCs is associated with enhanced tumorigenesis. Significant evidence has accumulated over the years that some tumor tissues and established cancer cell lines of neuroblastoma, AML, ovarian cancer, pancreatic cancer, and so on, have a small SP. It has also been significantly demonstrated that these SP cells, and not the non-SP cells, can self-renew, demonstrate enhanced chemoresistance, and form tumors *in vivo* (Haraguchi et al., 2006; Hirschmann-Jax et al., 2004; Kondo et al., 2004; Patrawala et al., 2005; Ponti et al., 2005; Szotek et al., 2006).

Along with separation and characterization of CSCs using cell surface marker through flow cytometry, CSC functional assay has been implicated in the isolation of CSCs to avoid sorting CSCs blindly based on some empirical stem cell markers. Clonogenic ability and sphere formation assays are key identifying features of CSCs *in vitro* relying on the anchorage-independent growth property of CSCs. These functional assays demonstrate isolation, enrichment and maintenance potential of CSC subpopulations in various types of cancers (Franken et al., 2006; Pastrana et al., 2011). Initially, the application of sphere-formation was described using neural crest stem cell culture conditions to isolate and expand tumor cells from neuroblastomas and bone-marrow metastases (Hansford et al., 2007). As low as ten cells, from the spheres of bone-marrow metastatic tumors are capable of forming metastatic tumors in a murine xenograft model (Hansford et al., 2007). Similarly, tumor cell invasiveness demonstrates a potential functional property useful for isolation/enrichment of CSCs from tumor cells. Indeed, using U87 glioma cell line, (Yu and Bian, 2009) found that the cells that invaded in response to the chemoattractant SDF-1 elicit a high tumor sphere formation and enhanced nestin and/or Oct-4 expression. Similarly, the invasive pancreatic cancer cells present in the lower chamber possess CSC characteristics of enhanced sphere formation and high expression of stem cell related genes (e.g., CD24, Oct, ESA, and resistance to gemcitabine treatment) (Wang et al., 2014). Thus, the most critical

step to understand the biological properties and consequences of CSCs depends on the accurate identification using reliable, sensitive and specific CSC biomarkers. Enriched CSCs can then further be employed to better understand perturbed signaling molecules and to develop therapeutic strategies targeting CSC population.

20.5 Pathological Significance of Cancer Stem Cells

CSCs are proposed to possess several distinct functional properties like self-renewal, multipotency, and apoptosis resistance similar to SSCs (Baccelli and Trumpp, 2012; Buczacki et al., 2011; Clarke and Fuller, 2006; Saigal and Bhargava, 2011). Moreover, accumulating evidence also indicates CSCs have high tumorigenecity with invasive and metastatic potential, which conveys resistance to chemo- and/or radio-therapies. Therefore, CSCs have been recognized as important factors of translational cancer research with potential in diagnostic, prognostic, and therapeutic targeting.

One of the most important properties of CSCs, is their unlimited ability of self-renewal which enables the maintenance of their sub-population in the tumor mass. Thus, unlike somatic stem cells, which respond to regional differences to initiate differentiation in a controlled fashion, CSCs continue to grow in an unrestricted manner leading to uncontrolled expansion of the tumor tissue (Al-Hajj and Clarke, 2004; Lobo et al., 2007). Moreover, a sub-population of CSCs may be maintained in a quiescent state or may proliferate at a very slow rate, such as those identified in acute myeloid leukemia and breast cancer (Buczacki et al., 2011; Pece et al., 2010). In addition, their response to the conventional therapies targeting fast proliferative cells is very poor and may lead to tumor recurrence due to potential shuttling between slow-cycling and active states (Aguirre-Ghiso, 2007). The slow proliferating property of these CSCs confers a survival advantage to these cells under stress conditions such as low nutrients, hypoxia, and so on.

Furthermore, CSCs have also been identified as the main source of tumorigenic sub-population in the tumor bulk. Subpopulation of glioma cells with the $CD133^+$ phenotype were demonstrated to recapitulate the heterogeneity of the parent tumor when transplanted in NOD/SCID mice (Singh et al., 2004). Similarly, (Li et al., 2007) demonstrated that as few as a hundred cells from the subpopulation of pancreatic cancer cells with $CD44^+/CD24^+/EpCAM^+$ phenotype were able to initiate tumor development *in vivo*. In another study, breast cancer cells expressing CD44 phenotype displayed enhanced stem cell-like characteristics and enhanced tumorigenic properties (Liu et al., 2011). Furthermore, the observation of enhanced apoptosis- and chemo-resistance in CSCs compared to the bulk of tumor may be directly associated with their enhanced tumorigenecity. Indeed, high expression of several anti-apoptotic proteins such as Bcl-2, survivin, and so on, has been observed in these CSCs. Furthermore, several studies have also identified CSCs to be significantly resistant to standard methods of chemotherapy. Leukemia stem cells have been observed to demonstrate resistance to Imatinib, a tyrosine kinase inhibitor (Oravecz-Wilson et al., 2009). Similarly, brain tumor cells with $CD133^+$ phenotype were found to be resistant to several chemotherapeutic drugs, such as carboplatin, temozolomide, etoposide (VP16), and paclitaxel (Taxol), as compared to $CD133^-$ cells. Thus, while a number of anti-cancer drugs are successful in eliminating the bulk of the cancers, it is postulated that the CSCs resistant to therapy survive and lead to the recurrence of cancer, indicating that these cells are capable of initiating malignancies. Various proteins and enzyme apparatus have been identified to confer chemoresistance in these CSCs. It has been observed that these cells have high expression of genes involved in apoptosis resistance (*BCL2*), DNA mismatch repair (*MGMT*) and multidrug resistance (*BCRP1*). Moreover, various ATP-binding cassette (ABC) transporters, breast cancer resistant protein 1 (BCRP1), multidrug resistant protein (MRP) etc. contribute to enhanced drug resistance observed in the CSCs.

Furthermore, recent evidence has underscored the importance of CSCs in migration and the establishment of distal macrometastasis. Epithelial to Mesenchymal transition (EMT) is an essential step in early metastasis which is also linked to CSCs. EMT requires the disruption of epithelial junctions and adhesion, induced

through the pleiotropic expression of transcription factors Snail, Slug, BMI-1, and Zeb1. Various factors responsible for maintenance of stemness of CSCs including Epidermal Growth Factor (EGF), Hepatocyte Growth Factor (HGF), Transforming Growth Factor β (TGF-β), Notch, Wnt/β-catenin and Hedgehog signaling pathways are observed to induce EMT. Activation of the EMT program in cells is observed to induce stem-cell phenotypes in certain cases. Ectopic expression of EMT inducers is also observed to upregulate CD44 and downregulate CD24 along with the alterations of other phenotypic markers in breast epithelial cells, which indicates the enhancement of CSC-like phenotypes (Asiedu et al., 2011; Battula et al., 2010; Hansford et al., 2007; Mani et al., 2008). It has also been demonstrated in breast cancer patients that the early-disseminated cancer cells present in the bone marrow exhibit putative CSC phenotypes. In fact, several potential CSC signatures have been observed as predictors for metastasis and formation of tumors in distant organs. For example, CSCs of $CD44^+$ phenotypes from primary breast tumors and metastatic sites exhibit high metastatic potential in xenograft experiments (Velasco-Velazquez et al., 2011). A chemokine receptor of SDF-1, CXCR4, has been demonstrated to provide a fundamental role in the metastasis of different types of cancer (Ben-Baruch, 2009; Gelmini et al., 2008; Singh et al., 2007) it is also an important biomarker for CSC identification. Tyagi and coworkers investigated the significance of PAK4, a serine/threonine kinase in the proliferation and survival of pancreatic cancer cells and further suggested its role in maintenance of the stem cell-like phenotypes in pancreatic cancer (Tyagi et al., 2014, 2016). Their study revealed that PAK4 is overexpressed in pancreatic CSCs. Silencing of PAK4 decreased the well-known stemness markers (CD24, CD44, and EpCAM) in PC cells, as well decreased the sphere-forming ability and sensitized the PC cells to gemcitabine toxicity (Tyagi et al., 2016). Thus, these studies highlight the importance of CSCs in the development and progression of cancer.

Another property of CSCs under exploration in relatively recent studies is their ability to evade host antitumor immunity (Di et al., 2010; Majeti et al., 2009; Schatton and Frank, 2009; Todaro et al., 2009). These studies demonstrate the ability of a fraction of cancer cells to impair the antitumor immune response to promote tumor growth (Dunn et al., 2002; Mapara and Sykes, 2004) and the ability of SSCs to modulate allo- and autoimmunity (Le and Ringden, 2007; Uccelli et al., 2008). For example, CSCs from glioblastoma cancer cells suppressed T-cell proliferation and the production of effector IL-2 and IFN-γ cytokines, in response to mitogen and anti-CD3/CD28 stimulation (Di et al., 2010). CSCs of $ABCB5^+$ phenotype from malignant melanoma subpopulations were observed inhibiting mitogen-mediated activation of peripheral blood mononuclear cells (PBMCs), relatively more efficiently as compared to the bulk populations (Schatton and Frank, 2009). Furthermore, these CSCs induced secretion of immunosuppressive IL-10 by the autologous PBMCs in co-culture (Schatton et al., 2010). Moreover, CSC subpopulations from colorectal cancer demonstrate resistance against cytotoxic lysis by γδ T cells, as compared to the bulk of colon cancer cells (Todaro et al., 2009). Several mechanisms have also been suggested by which these CSCs might exhibit enhanced antitumor immunoevasive properties. Studies have identified that these CSCs can evade immune system through the downregulation of tumor associated antigens (TAAs) (Khong et al., 2004), or by the downregulation or complete absence of MHC class I antigen expression (Schatton and Frank, 2009), consequently rendering the T-cells and CTLs unable to lyse the cells (Aptsiauri et al., 2007; Bubenik and Vonka, 2003; Khong et al., 2004). Therefore, the pathobiological significance of CSCs makes it eminent to successfully identify and develop therapies centered on the important signaling molecules regulating CSC-specific properties.

20.6 Pathways Regulating Cancer Stem Cells

The Oct4, Sox2, Nanog, KLF4 genes, Notch, Hedgehog, and Wnt pathways have specific roles in self-renewal and differentiation of normal stem cells and have now also been implicated in CSC maintenance and differentiation. These pathways are suggested to be regulated by the tumor microenvironment or CSC niche

which determines the self-renewal and differentiation potential of these CSCs (Figure 20.3/Plate 21). While the identification of nuclear transcription factors could previously be done only through destructive methods of intracellular immunostaining, western blotting or PCR, a recent article demonstrates a unique nanoparticle SmartFlare system for detection of mRNA transcripts in living cell using flow cytometry based detection and isolation (McClellan et al., 2015). Thus increasing stringency in the selection of CSCs from the total population. Next, we will discuss the function of some of transcription factors and signaling pathways, in no specific order of preference.

20.6.1 Oct4

The Oct3/4 gene encodes the POU family of transcription factors Oct3/4 or Oct4 (also known as POU5F1), which has an essential role in regulation of stem cells self-renewal and pluripotency (Hansis et al., 2000; Burdon et al., 2002). Oct4 is expressed exclusively in ESCs, during embryonic development and is also identified in all blastomeres; its expression is subsequently restricted to inner cell mass (ICM) (Pesce and Scholer, 2001; Scholer et al., 1990). Oct4 has multi-functional role in cancer and stem cell regulation (Gidekel et al., 2003). Moreover, it has been observed that Oct4 is expressed in human tumor cells, but its expression is not observed in somatic tissues. However, it is now well known that Oct4 is also expressed in ASCs. Upon downregulation of Oct4 activity in stem cells, terminal differentiation process in cells and stem cells occurs (Tai et al., 2005). It is observed when the breast epithelial, liver and spleen stem cells differentiate, a marked reduction in Oct4 expression is seen; however, on the contrary, upon forced upregulation of Oct4, the cells acquire stem cell-like features such as self-renewal. Emerging evidence demonstrates that Oct4 is highly expressed in human liver, spleen, breast, gastric, and kidney stem cells and in CSCs of breast, bladder, colon, prostate cancer tumor tissues, and cancer cells (Ponti et al., 2005; Amini et al., 2014). Thus it may be concluded, based on these observations, that ASCs expressing Oct4 could be direct targets for the initiation of cancer development. In distinction, reduced Oct4 expression confers the induction of apoptosis and survival of lung CSCs. Furthermore, Oct4 is also considered a good marker to identify the CSCs in breast cancer (Kim and Nam, 2011). Clinically, Oct4 expression in tumors is associated with disease progression, tumor metastasis and decreased survival of the patient (Chang et al., 2008). However, the molecular mechanism of Oct4 mediated-regulation of cancer stemness has not been completely elucidated.

20.6.2 Sox2

Sox2, a transcription factor belonging to sex-related high mobility group family of transcription factors (Stevanovic et al., 1994), has been observed to regulate maintenance of undifferentiated ESCs (Masui et al., 2007). Sox2 is one of the important transcription factors capable of reprogramming the differentiated somatic cell into induced pluripotent stem cells (Park et al., 2008). Elevated Sox2 expression is detected in many type of cancers such as breast, ovarian, lung, prostate, and squamous cell carcinoma (Chen et al., 2012; Jia et al., 2011; Zhang et al., 2012). Sox2 target genes have demonstrated direct responsibility for tumor invasion, survival, proliferation, and adhesion in skin squamous cell carcinoma (Boumahdi et al., 2014). Moreover, the Sox2 transcriptional activity is also indicated to be responsible for maintenance of CSC phenotypes in breast cancer (Wu et al., 2012), pancreatic cancer (Herreros-Villanueva et al., 2013), for example evasion from apoptosis, enhanced tumorigenesis and self-renewal capacity (Xiang et al., 2011). A strong correlation of sphere forming efficiency in Sox2 expression is also observed in pancreatic adenocarcinoma (Xiang et al., 2011). The expression of other stem cell markers, for example CD44 and ALDH, are also directly associated with Sox2 transcriptional activity. Moreover, forced expression of Oct4 in Sox2-null cells leads to the maintenance of pluripotency in these cells; this suggests that one of the important roles of Sox2 is to control the expression of Oct4 and to subsequently maintain stemness (Masui et al., 2007). Furthermore, Sox2 is observed

to enhance self-renewal capacity and to regulate dedifferentiation and EMT in cancer cells (Herreros-Villanueva et al., 2013). Over expression of Sox2 reduces the epithelial maker E-Cadherin, with a concurrent increase in the Snail, Slug family of zinc-finger transcription factors (Herreros-Villanueva et al., 2013). Targeting of Sox2 gene expression inhibits self-renewal and tumorigenecity of CSCs, the root cause of the tumor progression, drug resistance and relapse.

20.6.3 Nanog

Nanog, an important transcription factor for maintaining pluripotency, is expressed in embryonic stem cells but not in differentiated cell (Hatano et al., 2005). Nanog over expression is detected in many human cancers including pancreatic (Lu et al., 2013), colon (Zhang et al., 2013), leukemia (Eberle et al., 2010), breast (Jeter et al., 2009), lung (Du et al., 2013), ovarian (Jeter et al., 2009; Zhang et al., 2008), hepatocellular (Shan et al., 2012), and brain (Elsir et al., 2014); also it has been identified to have essential role in human tumor progression and poor patient prognosis (Kregel et al., 2014; Meng et al., 2010; Siu et al., 2013). Nanog is believed to be a biomarker for CSC in hepatocellular carcinoma and the maintenance of self- renewal potential in liver CSCs (Shan et al., 2012). Its over-expression is also identified in glioblastoma CSCs while regulating tumor initiation and tumor development through activation of glioma-associated oncogenes (Bae et al., 2010; Zbinden et al., 2010). Nanog expression also correlates with the stage of disease and prognoses of patients with cervical neoplasia. High Nanog expressing cells demonstrate stem cell-like evasion from immune attack and anti-apoptotic properties. Most importantly, Nanog transcriptional activity promotes the stem cell-like phenotype and EMT in hepatocellular carcinoma, through activation of STAT3/Snail signaling pathway (Yin et al., 2015) and in recruiting the T-cell leukemia through the Akt signaling axis (Noh et al., 2012). Moreover, Nanog expression is correlated with a small population of CSC marker positive cells and has a role in direct regulation of stem-like phenotype, proliferation, migration, invasion, drug-resistance, and metastasis-associated properties (Choi et al., 2012; Han et al., 2012; Ibrahim et al., 2012; Jeter et al., 2011; Siu et al., 2013). However, the role of Nanog in CSCs and its associated markers is still not clear. Nanog and its role in maintaining stemness and cancer propagation should be further elucidated for therapeutic potential and for targeting Nanog in cancer and cancer stem cells.

20.6.4 KLF4

Kruppel-like factor 4 (KLF4) is a family of conserved zinc finger DNA binding transcription factors (Schuh et al., 1986). KLF4, expressed in variety of human tissues, has diverse physiological functions including proliferation, differentiation, inflammation, pluripotency, homeostatic maintenance, apoptosis, reprogramming, and migration. Alteration in gene expression in response to aberrant KLF4 levels, may lead to many diseases including cardiovascular disease, metabolic disorder and cancer. In cancer, KLF4 acts as a transcriptional repressor or activator and can either function as a tumor oncogene or tumor suppressor depending on the tumor, tumor type and tumor stage (Chen et al., 2001; McConnell et al., 2007; Rowland et al., 2005; Rowland and Peeper, 2006; Shields et al., 1996). KLF4 is observed to act as a tumor suppressor in colorectal cancer and gastric cancer; however, its expression does not correlate with tumor stage (Kanai et al., 2006; Xu et al., 2008; Zhao et al., 2004). KLF4 was first demonstrated as an oncogene in late 1990s, when overexpression was observed in many tumors including squamous cell carcinoma (Foster et al., 1999), breast (Foster et al., 2000), and colon (Dang et al., 2003). KLF4 primarily regulates telomerase activity in ASCs and CSCs, indicating that KLF4 has long term role in proliferation capability of stem cells. Promotion of EMT and stem cell phenotypes has been established as a result of KLF4 and TGF-β in breast CSC (Yu et al., 2011). KLF4 also enhances spheroid formation and maintains the CSC-like cells in colon cancer. In contrast, inhibition of KLF4 leads to a decrease in the colon CSC marker expression (Leng et al., 2013). KLF4 expression is

upregulated by ZEB1 and controls the CSC properties in pancreatic cancer (Wellner et al., 2009). Additionally, miRNA-7, a brain-specific miRNA, inhibits brain metastasis through down regulation of KLF4 in brain CSCs (Okuda et al., 2013). Furthermore, KLF4 is the one of four factors used to reprogram differentiated cells into induced pluripotent stem cells and is essential for maintaining stem cell properties (Ding et al., 2015; Yamanaka, 2007). Therefore, understanding the KLF4 role on tumor development and CSC regulation provides a potent therapeutic target strategy for cancer disease.

20.6.5 Notch

The Notch signaling pathway plays an important role during development of organs, proliferation, differentiation and apoptosis (Miele and Osborne B., 1999). Four Notch transmembrane receptors (Notch1, Notch2, Notch3, and Notch4) and five ligands (Delta1, Delta3, Delta4, Jagged-1, and Jagged-2) in mammalian system have been identified (Dunwoodie et al., 1997). Notch signaling activation requires a cascade of proteolytic cleavages; first, cleavage by tumor necrosis factor-α- converting enzyme (TACE) at an extracellular transmembrane protein and second, cleavage by γ-secretase complex at the side of the Notch intracellular domain (NICD). NICD then translocates to the nucleus and binds to CSL (CBF1, suppressor of hairless and lag-1) and other co-factors, subsequently, activating the downstream target genes (Miele and Osborne, 1999). Several studies demonstrate oncogenic functions of Notch signaling in several different cancers, such as head and neck, lung, colon, cervical, acute myeloid, renal carcinoma, prostate and pancreatic cancer (Mysliwiec and Boucher, 2009; Ristorcelli and Lombardo, 2010; Wang et al., 2008). Although Notch also regulates the early development of pancreatic epithelial progenitor stem cells and their differentiation, interestingly, the Notch receptor and their target genes are also over expressed in pancreatic cancer, which indicates a reactivation of Notch signaling in pancreatic cancer pathogenesis (Ji et al., 2009; Mysliwiec and Boucher, 2009). Furthermore, Notch regulated genes are believed to play important role in CSCs and CSC fate (Phillips et al., 2007); for example, Notch signaling regulates the breast CSCs through activation of Jagged1 and Jagged2 ligands, also elevated expression of Notch target genes in breast CSCs is observed (Zang et al., 2007). Significant cross-talk between Notch signaling and other transcription and growth factors has also been shown. Notch promotes the EMT through direct upregulation of target, Slug (Timmerman et al., 2004). Increased expression of Notch1 is also detected in prostate CSCs (Domingo-Domenech et al., 2012). Hence, Notch signaling may be developed as a potent target for CSCs therapeutic targeting.

20.6.6 Wnt

The Wnt signaling pathway comprises a large family of proteins and evolutionary conserved signaling pathways from invertebrate to vertebrate. Wnt signaling plays a varied role during embryonic development including stem cell maintenance, proliferation, and differentiation, (Cadigan and Nusse, 1997). The Wnt ligand binds to the fizzed or LRP5/6 co-receptors activates β-catenin from the dissociation of the protein APC/CK1/GSK3β complex, such as casein kinase 1 (CK1) adenomatous polyposis coli (APC) 2 kinases, and glycogen synthase kinase 3β (GSK3-β) and Axin2. Wnt dysregulation leads to expansion of stem cells in many tissues which is highly associated with tumorigenesis (Batlle et al., 2005). Wnt signaling influences mammary gland stem cells maintenance during developmental stages (Inman et al., 2015). Aberrant expression of Wnt ligands in stem cells and cancer has been observed (Ring et al., 2014). Furthermore, several CSC markers are target genes for the Wnt pathway, such as, CD44 (Wielenga et al., 1999), CD24 (Shulewitz et al., 2006), CD133 (Katoh and Katoh M., 2007), ABC cassette genes (Yamada et al., 2000), and EpCAM (Munz et al., 2009). Moreover, Wnt/β-catenin is also involved in transcription of drug resistance-associated genes, that is, MDR-1, ABCG2, ABCA3, and BRCP1 (Heidel et al., 2012; Ring et al., 2014; Wang et al., 2010). Wnt/β-catenin-dependent EMT and pancreatic CSC maintenance play an important role in pancreatic cancer

pathogenesis (Heidel et al., 2012; Stemmer et al., 2008). High levels of Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) in breast CSCs, through activation of Wnt/β-catenin pathway enhances breast cancer development and maintains the tumor-initiating cells self-renewal capacity (Yang et al., 2015). In addition, focal adhesion kinase (FAK), and Wnt axis maintains the breast CSCs self-renewal in breast cancer (Williams et al., 2015). Thus the Wnt pathway may be a potent target for CSC therapies. However, since the Wnt pathway regulates many biological functions in adult tissues, such as homeostasis and repair, successful targeting of the Wnt pathway in CSCs, without interfering with their normal function needs to be further explored.

20.6.7 Hedgehog

Hedgehog (Hh), initially identified in *Drosophila*, is a highly conversed pathway in vertebrates. Hh has a critical role in embryonic development from invertebrates to mammals and regulates diverse biological functions such as proliferation, migration, differentiation, cell fate and body segment polarity (Ingham and McMahon, 2001; Nusslein-Volhard and Wieschaus, 1980). Hh, a secreted protein that binds to the transmembrane receptor Patched (PTCH1), leads to its internalization resulting in the activation of the transmembrane protein Smoothened (Smo). Smo activates the Gli family of zing finger transcription factors, then translocates into nucleus inducing the transcription of the target genes (Denef et al., 2000). Hh promotes tumorigenesis in different cancers such as gastric, prostate, pancreatic, ovarian, lung, liver, brain and skin (Barakat et al., 2010; Teglund and Toftgard, 2010). Furthermore, alteration of the Hh signaling pathway and their components lead to enhanced survival and metastasis in cancer cells (Evangelista et al., 2006; Nieuwenhuis and Hui, 2005;). It is now observed that Hh signaling plays a significant role in CSC maintenance in breast, colon, pancreatic, myeloma, glioblastoma, and leukemia (Bar et al., 2007a; Justilien et al., 2014; Liu et al., 2006; Varnat et al., 2009). Hh directly induces the CSC phenotype through regulation of expression of CSC markers and also through the induction of drug resistance by regulation of MDR1 and ABCG2 gene expression (Huang et al., 2012; Olive et al., 2009; Sims-Mourtada et al., 2007; Singh et al., 2012). Moreover, inhibition of Hh leads to decreased self-renewal, metastasis and tumor growth potential in different cancers (Bar et al., 2007a; Justilien et al., 2014; Liu et al., 2006; Varnat et al., 2009). Hh ligands and Gli activity are high in breast and glioblastoma CSCs, where they enhance stemness and tumorigenecity by regulation the BMI-1 (Liu et al., 2006). Hh signaling also regulates a broad range of hematological malignancies by direct regulation of HSC, or indirectly through the regulation of the stromal compartment (Campbell and Copland, 2015). A combination of inhibition strategies targeting Hh and mTOR pathways, demonstrate decreased self-renewal and chemoresistance through the downregulation of pluripotent transcription factors like, Oct4, Nanog, Sox2, KLF4, and Gli (Sharma et al., 2015). Thus, in a clinical study inhibition of Hh signaling in CSC should improve patient survival and long term outcome.

20.6.8 Micro RNAs

MicroRNAs (miRNAs or miRs) are one of the most important post-transcriptional regulators of gene expression. By inducing mutants, Dicer, an important mediator of the miRNA processing, miRNAs regulate stem cell differentiation (Kanellopoulou et al., 2005). Further, research on the role of miRNAs in CSCs demonstrates that that a number of miRNAs, for example, miR-21, let-7, miR-21, miR-320, miR-34, and so on, modulate self-renewal, differentiation, and survival of CSCs (Domingo-Domenech et al., 2012; Hsieh et al., 2013; Ji et al., 2009; Shimono et al., 2009; Yu et al., 2007). Based on their role in tumors, miRNAs are also classified as oncogenic or tumor suppressors (Chen, 2005). Several oncogenic miRNAs, such as miR-17-92, miR-21, miR-135 family, are overexpressed in CSCs (Nagel et al., 2008; Uziel et al., 2009). Simultaneously, the expression of several tumor suppressor miRNAs is observed to be decreased in cancer compared to

expression in normal tissues. Tumor suppressor miRNAs, such as Let-7, miR-15a, miR-34, miR-128, and miR-16 may prevent tumor development by negative regulation of oncogene expression, self-renewal, and differentiation of CSCs (Cimmino et al., 2005; Godlewski et al., 2008; Lee and Dutta, 2007; Yu et al., 2007). Furthermore, miRNAs also regulate key properties associated with CSCs such as cell-cycle and differentiation, survival mechanisms, resistance to stress, EMT, migration and invasion, all of which contribute to enhanced tumor progression and maintenance.

Yu et al. presented the first evidence for the role of miRNA in breast CSCs. They enriched breast CSCs expressing the phenotype $CD44^{+}/Cd24^{-/lo}$, through consecutive serial passaging of breast cancer cell line in mice and with the simultaneous administration of chemotherapy (Yu et al., 2007). These enriched breast CSCs expressed low levels of several miRNAs, such as Let-7, miR-107, miR-128, miR-16, and miR-20. In another study on breast CSCs, the same group demonstrated Let-7 along with miR-30 regulates stemness in CSCs. Ectopic expressions of Let-7 and miR-30 inhibited self-renewal and mammosphere formation in breast CSCs. Similarly, miRNA expression in breast cancer cells with high ALDH activity revealed downregulation of miR-93 and the Let-7 family, along with an upregulation of miR-205 and miR-22 (Ibarra et al., 2007).

Furthermore, $CD133^{+}$ glioblastoma multiforme (GBM) cells are observed to have decreased expression of miR-451, miR-16, miR-486, miR-425, miR-185, and miR-107. $EpCAM^{+}/AFP^{+}$ CSCs from hepatocellular carcinoma are found to express a unique miRNA signature with upregulation of the miR-181 family members and miR-17-92 clusters (Ji et al., 2009). Later, miR-128 demonstrated inhibition of glioma stem cell proliferation and self-renewal through the targeting of BMI-1 (Jin et al., 2014). Similarly, in prostate CSCs, Let-7b and miR-34a are found to be significantly underexpressed (Liu et al., 2012); miR-34a is further corroborated as downregulated in $CD44^{+}$ prostate CSCs taken from 20 patient samples (Liu and Tang, 2011). Again, ectopic expression of miR-34a in prostate CSCs suppressed prostasphere formation, decreased invasion and migration of $CD44^{+}$ prostate CSCs. Thus, further investigations of miRNAs and CSCs provide additional understanding of the regulation of CSCs by miRNAs and potential targeting strategies.

20.7 Therapeutic Strategies Targeting Cancer Stem Cells

The most common methods to treat cancers include chemotherapy and radiotherapy, which target highly mitotic and rapidly dividing cells (Figure 20.4/Plate 22). These therapies overlook slow-dividing CSCs that evade treatments and a sub-population of remnant cells, representing CSCs, re-establish tumor. Therefore, elimination of the highly resistant CSC subpopulation is necessary to treat malignancy. The current strategies targeting CSCs include disrupting mechanisms that impart stability and resistance to CSCs, that is relative dormancy/slow cell cycle kinetics, high capacity for DNA repair, high expression of multiple drug resistance membrane transporters (e.g., ABC transporters), high expression of anti-apoptotic proteins, the microenvironment (hypoxia, acidosis, etc.), the presence of detoxifying enzymes, and distinct oncogenic cascades (e.g., the hedgehog, notch, Wnt/β-catenin pathways, NF-κB pathway). Thus, novel treatment strategies may include a combination of traditional chemo-radiotherapy with new strategies targeting CSCs that will appropriately and effectively prevent tumor relapse providing a potentially less toxic cancer therapy. Here we discuss the latest strategies that may target and eliminate CSCs.

20.7.1 Targeting CSC-Specific Markers

Several studies have demonstrated that by exploiting the ligands or antibodies against CSCs specific surface markers successful inhibition of CSCs can be achieved. The most notable example is gemtuzumab ozogamicin, a humanized anti-CD33 mouse monoclonal antibody conjugated to the cytotoxic agent calicheamicin widely

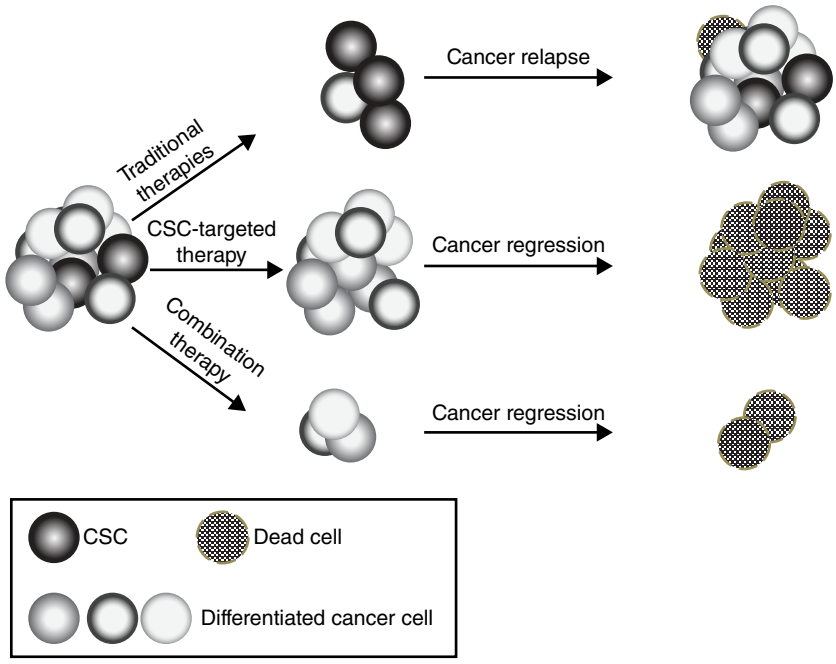

Figure 20.4 (Plate 22) A general overview of the concept of cancer stem cell-targeted therapy. *CSCs have been observed to be resistant to almost all of the current chemo and radiation therapies employed in the clinics. Thus, while an initial reduction in tumor burden is observed with these therapies, a relapse is almost always detected in these patients due to the surviving CSCs. Therefore, cancer therapies specifically targeting CSCs or a combination therapy targeting both the CSC subpopulation and differentiated cells have been investigated to enhance therapeutic outcomes. (See insert for color representation of the figure.)*

used to treat AML (Curiel, 2012), CD44 (Jin et al., 2006), IL-3R (Jin et al., 2009), CD47 (Chao et al., 2011), and the immunoglobulin mucin TIM-3 (Kikushige et al., 2010), exhibit differential expression in normal stem cells and CSCs, and serve as appropriate targets for treatment of AML. For breast cancer treatment, CD44 surface marker has been exploited. An anti-CD44 antibody-conjugated gold nanorod, specifically targets CSCs; when further challenged with infrared light, CSCs are eradicated as a result of high temperature (Alkilany et al., 2012). CD133 (prominin-1, PROM1) is also expressed in CSCs of different cancer types and targeting CD133 using short hairpin RNAs in human metastatic melanoma decreased its growth, metastasis and sphere forming ability (Rappa et al., 2008). Similar targeting CD133 in glioblastoma neurospheres resulted in its diminished self-renewal ability and tumorigenecity (Brescia et al., 2013). Monoclonal antibodies against CD133 were used to treat human malignant melanoma cells, which exhibited a dose-dependent cytotoxic effect on these cells. *In vitro* studies using an anti-human CD133 antibody conjugated to a potent cytotoxic drug (monomethyl auristatin F) report an effective inhibition in the growth of Hep3B hepatocellular cancer cells and KATO III, gastric cancer cells (Smith et al., 2008). Wang et al. (2011a) devised a protocol to photothermolyse the glioblastoma stem-like cells. They used carbon nanotubes conjugated to an anti-CD133 monoclonal antibody to irradiate CSCs, with near infrared laser light, leading to lysis and death of the cells.

ALDH can act as drug-detoxifying enzymes and thus confer therapeutic resistance. The Aldefluor assay, ALDH-activated fluorescent substrate is used to isolate CSCs displaying high ALDH activity (Kitamura et al., 2009). In human breast cancer a subpopulation of CSCs expressing $ALDH^{high}/CD44^{+}$ marker is identified (Croker and Allan, 2012; Croker et al., 2009). These CSCs are highly resistant to chemotherapy

(doxorubicin/paclitaxel) and radiotherapy. Treatment of this CSC subpopulation with a specific ALDH inhibitor, like diethylaminobenzaldehyde (DEAB) or all trans retinoic acid (ATRA), made them more susceptible to cancer therapies. DEAB exhibited long-term sensitization effect; thus, blocking ALDH activity offers a promising strategy to target CSCs.

The ATP-driven pumps or drug transporters are overexpressed in both normal and tumor stem cells. These pumps protect the SCs from xenobiotic toxins and confer chemoresistance to these cells. MRPs/ABCC, BCRP/ABCG2, and P-gp/ABCB1 are the drug effluxing pumps; the CSCs expressing them are designated as side population (SP). Verapamil is the first inhibitor of the P-gp efflux pump; it is often employed for SP analysis, as it blocks the exclusion of Hoechst dye (Tsuruo et al., 1981). Verapamil in combination with other antitumor drugs such as doxorubicin (Dox), paclitaxel or vincristine has provided a promising therapeutic strategy for treatment through CSCs targeting. Methylene blue, another inhibitor of P-gp, when administered with Dox into BALB/c mice bearing tumors, was significantly able to reduce tumor growth and improve survival (Khdair et al., 2010). Twelve high drug efflux cancer cell inhibitors were derived from 1280 pharmacologically active compounds, which through *in vivo* and *in vitro* assays demonstrate suppression of MDR, thus inhibiting SP, increasing the efficacy of chemotherapy and reducing tumorigenecity of lung cancer (Xia et al., 2010). Several inhibitors of P-gp and MRP1 are currently in clinical trials. MS-209 overcomes drug resistance in breast cancer and other solid tumors. Tariquidar in combination with docetaxel is under clinical trials for the treatment of recurrent and metastatic ovarian, cervical, lung, and kidney tumors (Amiri-Kordestani et al., 2012); several other combinations of tariquidar with Dox, etoposide, mitotane, and vincristine are being tested in different types of adrenocortical cancers (Amiri-Kordestani et al., 2012). Daily treatment of rat glioma cell lines with curcumin decreased the SP cells, emphasizing the therapeutic potentials of phytochemicals for targeting CSCs (Fong et al., 2010). The major drawback associated with the targeting of drug efflux pumps is that these transporters are present on both CSCs as well as normal stem cells; they play important role in maintaining the blood-brain barrier, so there is need to develop therapies that specifically target CSCs and do not affect the functioning of normal stem cells.

20.7.2 Targeting CSC-Specific Molecular Signaling Pathways

The binding of Hh protein to its Patched (Ptch) transmembrane protein activates the Hh pathway, that then regulates the genes implicated in many cellular processes such as proliferation, survival, metastasis, and pathway auto-regulation (Maugeri-Sacca et al., 2011; Varjosalo and Taipale, 2008; Corbit et al., 2005). The aberrant activation of this pathway is also associated with tumorigenesis. The Hh pathway is implicated in the maintenance of CSCs in pancreatic cancer, gastric cancer, colorectal cancer, and many other cancers and it plays an important role in imparting resistance to treatment (Merchant and Matsui, 2010; Song et al., 2011; Tang et al., 2012). Small molecule Hh antagonists inhibit systemic metastasis in mice with orthotopic xenografts from human pancreatic cancer cell lines (Feldmann et al., 2008a, 2008b). These antagonists reduce the population of ALDH-positive cells. Cyclopamine and GDC-0449 (Vismodegib) target and inhibit the signaling molecule smoothened (SMO) and they are shown to inhibit both *in vivo* and *in vitro* growth, invasion and metastasis of breast, prostate, pancreatic, and brain cancers (Bar et al., 2007b; Feldmann et al., 2008b; Karhadkar et al., 2004; Ramaswamy et al., 2012). Cyclopamine in combination with gemcitabine reduced the population of $ALDH^{high}$ in pancreatic cancer, while its combination with temozolomide (TMZ) reduced the population of CSCs in glioma (Clement et al., 2007; Feldmann et al., 2008b). For tumors with molecular lesions downstream of SMO, other agents targeting glioma-associated oncogene homolog (Gli) proteins may be used in combination with SMO inhibitors (Ng and Curran, 2011). Epigallocatechin gallate or epigallocatechin-3-gallate (EGCG) potentially inhibits various components of sonic hedgehog pathways including SMO, Ptch, Gli1, and Gli2 (Tang et al., 2012). It also targets the transcriptional activity of Gli. Combination of quercetin with EGCG, synergistically targets the self-renewal

capacity of CSCs, through attenuation of TCF/LEF and Gli activities. Thus, targeting the SHh pathway offers a very promising therapeutic strategy to target CSCs.

As the Notch pathway is found to be over-activated in variety of cancers, the use of monoclonal antibodies targeting its signaling offers a promising strategy to eliminate CSCs (Lobry et al., 2011; Pannuti et al., 2010; Qiao and Wong, 2009; Wang et al., 2011b; Xia et al., 2012). Notch1 inhibition significantly reduced brain metastasis, by reducing the subpopulation of $CD44^{+}CD24^{-/low}$ cells from a breast cancer cell line (McGowan et al., 2011). Gamma-secretase inhibitors (GSIs) demonstrate inhibition of the notch signaling pathway, by blocking the proteolytic process of the formation of Notch intracellular domain (NICD) (Olsauskas-Kuprys et al., 2013). In glioblastomas, the use of GSIs not only reduced *in vitro* neurosphere growth and clonegenicity, but also decreased the subpopulation of CSCs, as well as reduced the *in vivo* growth of tumor (Fan et al., 2010). Similarly, GSI MRK-003 is found to eliminate CSCs, inhibiting self-renewal and proliferation in breast cancer cell lines (Kondratyev et al., 2012). However, the non-selective nature of GSIs enhances their potential toxicity in humans (Wang et al., 2011b); therefore, the development of a more precisely targeted therapy to disrupt this signaling pathway is required.

The oncogenic mutations of β-catenin or mutations inactivating tumor suppressor activity of APC (adenomatous polyposis coli) result in a dysregulated Wnt/β-catenin pathway inducing neoplastic proliferation of CSCs. Nonsteroidal anti-inflammatory drugs (NSAIDS) or other natural compounds and cAMP response-element binding protein (CBP)/β-catenin antagonist ICG-001 may potentially target the Wnt/β-catenin signaling pathways (Henderson, et al., 2010). Biologic inhibitors of the Wnt/β-catenin signaling pathway include monoclonal antibodies, small interfering RNAs (siRNAs) and recombinant proteins against wnt1/2, WIF1 (Wnt inhibitory factor 1), and sFRPs (secreted frizzled-related proteins). Both *in vivo* and *in vitro* targeting of Wnt signaling pathway have been conducted (Takahashi-Yanaga and Kahn, 2010). In another study, Teng et al. knocked down the expression of β-catenin using RNA interference technology, resulting in attenuation of CSCs properties, downregulation of cyclin D1, OCT-4, proliferation, colony formation, migration, and drug resistance (Teng et al., 2010). However, the targeting agents can have undesirable effects on normal stem cells so it is required that it is appropriately modified and combined with other agents, so that only CSCs are targeted and its deleterious effects are minimized.

20.7.3 CSC-Related Immunotherapy

The immune system is a tremendously complex organization of cells which work in a coordinated fashion, to protect, defend, and maintain homeostasis. Cancer cells effectively evade host immune surveillance, induced by a variety of genetic and pathological deficiencies. This complexity establishes CSC-related immunotherapy as a challenging area of cancer research. Innate immune responses employing the use of Natural Killer (NK) cells and gamma delta (γδ)T-cells are being investigated for targeting of CSCs. Glioma stem cells (GSCs) are targeted by both allogenic and autologous IL-2 (or IL-15) activated NK cells (Castriconi et al., 2009). In, colorectal cancers it was shown that allogeneic NK cells can recognize and selectively kill colorectal carcinoma-derived cancer-initiating cells (Tallerico et al., 2013). Another important innate immune effector cell, Gamma delta T-cells (γδ T) cells are employed to target CSCs. They constitute 1-5% of the circulating lymphocytes and are of the Vγ9Vδ2 phenotype (Pan et al., 2015). Vγ9Vδ2 T-cells in combination with the drug zoledronate elicit innate-mediated immune responses killing sphere-forming neuroblastoma cells and human colon CSCs (Nishio et al., 2012; Todaro et al., 2009). Stimulation of $CD8^{+}$ T-cells generate cytotoxic T lymphocytes (CTLs) that can either exert direct contact-mediated cytotoxicity or secrete effector cytokines such as IFN-γ or TNF-α (Huang et al., 2007). Activated $CD8^{+}$ T-cells play an important role in memory acquisition and can potentiate a more potent and faster response upon cancer relapse or development of metastasis (Ahlers and Belyakov, 2010). $CD8^{+}$ cytotoxic T lymphocyte clones specific for specific for minor histocompatibility antigen resulted in the successful elimination of human acute myeloid leukemia

stem cells and tumor in NOD/SCID mice (Bonnet et al., 1999). Another promising strategy to target CSCs includes the use of dendritic cells (DCs) to initiate tumor-specific T-cell responses. When CSC-DCs primed with ALDH antigen and subsequently administered as a vaccine in immunocompetent mouse tumor models, results yielded prevention of lung metastasis of melanoma cells, and inhibition of growth of squamous carcinoma cells, as compared to immunization with their non-CSC tumor bulk (Ning et al., 2012). In a similar study, the breast CSC-DC derived vaccine migrated to the spleen and efficiently initiated CTL response by activating CD8+ and CD45+ T-cells. CD44v, with a monoclonal antibody, 1.1ASML, in rat pancreatic adenocarcinoma significantly reduced metastasis to lymph nodes as well as lung tissue (Seiter et al., 1993). The use of monoclonal antibodies against CD44 also decreased melanoma metastasis and increased survival of SCID mice (Guo et al., 1994). Moreover, anti-CD44 antibody, block the trafficking of stem cells to their supportive microenvironment, thus altering the stem cell fate (Jin et al., 2006), and the administration of this antibody to SCID/NOD mice selectively eradicates AML CSCs (Frank et al., 2010). Humanized antibody, against CD44, reduced the growth of BxPC3 pancreatic cancer xenografts by 80% (Deonarain et al., 2009). Monoclonal antibodies, targeting highly immunogenic amino acid residues, against CD133 have been generated for targeted therapy (Swaminathan et al., 2010). A bispecific antibody (BsAb), against CD3 and CD133, has been developed and it binds to cytokine-induced killer (CIK) cells as effector cells that can efficiently target $CD133^{high}$ CSCs. These conjugated CIK cells were more efficient in killing $CD133^{high}$ pancreatic and hepatic cancer cells as compared to the CIK cells bound to anti-CD3 alone (Huang et al., 2013; Pan et al., 2015). Activated T-cells armed with bispecific antibody (HER2Bi) against CD3 and HER2 are reported to mediate cytotoxicity against both high and low HER2-expressing breast cancer cell lines (Sen et al., 2001). Thus the administration of HER2Bi armed T-cells inhibit the growth of PC3 tumors, as well as increased the survival of metastatic breast cancer patients (Davol et al., 2004; Pan et al., 2015).

20.7.4 Targeting CSC Microenvironment

In order to maintain CSC characteristics, these cells require a microenvironment or niche that provides appropriate signals, through the activation of various signaling pathways lending it the typical characteristics of self-renewal, drug resistance, quiescence state and the normal homeostatic processes, such as inflammation, EMT, hypoxia and angiogenesis (Cabarcas et al., 2011). For example, in colon cancer the Wnt pathway is found to define colon CSCs and is regulated by the micro-environment (Vermeulen et al., 2010). Similarly, a perivascular niche is found to be critical for the maintenance of brain CSCs (Calabrese et al., 2007). Another important pathway playing a role in rendering chemoresistance and increased growth is CXCL12/CXCR4 signaling that involves the interaction between stromal cell derived factor-1 (SDF-1/CXCL12) and its receptor CXCR4 present on cancer cells. The interactions between these cells provide them with more protective homing grounds, which is appropriate for the maintenance of CSCs. CXCR4 antagonists such as Plerixafor (AMD3100) and T14003 analogs disrupt these interactions; therefore, the adhesive and protected CSCs are then mobilized and are accessible to cytotoxic drugs. Different approaches targeting the CXCL12-CXCR4 axis are under considerations and CXCR4 antagonists are under clinical trials for the treatment of leukemia (Burger and Peled, 2009; Konopleva et al., 2009). Hypoxia plays an important role in tumor progression and it is found to play an important in controlling CSCs (Seidel et al., 2010). The anti-angiogenic therapy is speculated to induce hypoxia enriching CSCs, thus, this niche may potentially be responsible for conferring radioresistance to the CSCs (Morrison et al., 2011). Intratumoral hypoxia generated by the use of antiangiogenic agents, such as sunitinib. and bevacizumab in breast cancer is observed to enrich CSCs in its niche (Conley et al., 2012). VEGF is associated with increased angiogenesis and is important for microvasculature formation and tumor growth. Bevacizumab, which targets VEGF is used to treat mouse glioblastoma and demonstrates a dramatic reduction in the number of glioblastoma stem cells (Burkhardt et al., 2012). Similarly, U87 glioma cell xenografts, when treated with bevacizumab, reportedly decreased angiogenesis, tumor

growth, and reduced the number of $CD133^+$/$nestin^+$ cells that are important tumor-initiating cells (Calabrese et al., 2007). Thus, it is important that antiangiogenic agents must be combined with a CSC-targeted drug in order to prevent cancer re-occurrence.

The MDSCs secrete pro-angiogenic factors; induce the production of matrix metalloproteinases and chemoattractants, which facilitate the metastasis of tumor cells. MDSCs demonstrate an increase in the population of $ALDH^{high}$ CSCs in pancreatic cancer (Panni et al., 2014), promote CSCs and EMT through the IL6-Stat3 signaling pathway (Marigo et al., 2010; Wang et al., 2009), enhance the expression of CSC gene expression, sphere formation and metastasis in ovarian cancer patients (Cui et al., 2013). Targeting MSDCs through immunotherapy offers a promising technique, ultimately targeting CSCs. Monocytes and macrophages recruited to breast tumor secrete several cytokines IL-1, IL-16, and IL-18 in the microenvironment that promote self-renewal of CSCs (Korkaya et al., 2011). Blocking IL-8 receptor, CXCR1, by an antibody reduced the population of CSCs in human breast cancer cell lines, as well as, induced apoptosis in the bulk tumor population via FASL/FAS signaling (Ginestier et al., 2010). IL-6 is another important regulator of CSC self-renewal (Iliopoulos et al., 2009; Liu et al., 2011; Rokavec et al., 2012). IL-4 blockade by an antibody inhibits JAK1, STAT3, and OCT-4 (Kim et al., 2013) expression, thus regulating CSCs and is an important target for immunotherapy. Two major immunoinhibitory pathways named programmed cell death (PD-1)/PD-L1 axis and the CTL antigen 4 (CTLA-4)/B7 axis provide an immune-suppressive niche conveying protection to CSCs. In melanoma cancer, initiating CSCs are identified and designated as MMICs. These have high expression of chemoresistance determinant ABCB5 and expressed PD-1 and B7.2, while the ABCB5- cells have low expression levels of PD-L1. Antibodies against PD-1 and PD-L1 have shown clinical benefits in melanoma, lung cancer, and Hodgkin's disease (Ansell et al., 2015; Sharma et al., 2011; Topalian et al., 2012). PD-L1 expression is associated with downregulation of T-cell responses; therefore, its blockade might enhance the effective T-cell responses. So combined immunotherapy targeting immune checkpoints, as well as, vaccines may offer promising therapies effective in targeting cancer.

20.8 Conclusion and Future Directions

CSCs are a small proportion of cancer cells that are present within tumors or hematological malignancies. These specialized cells have capacity for self-renewal and unlimited proliferation. Moreover, CSCs play a major role in tumor initiation, development, progression and resistance to radiation and chemotherapeutic regimens. Although CSCs have now been well characterized, but still methodologies for their accurate identification remains a major challenge. CSCs are being isolated based on the presence of phenotypic surface markers. These CSCs may either express a single cell or multiple surface markers. Several stemness associated markers have been identified that are potentially being used for the isolation and characterization of stem cells. These markers include CD133, CD44 CD24, EpCAM, CD117, *ALDH1*, and so on. In addition, the biological significance of many of these markers has also been demonstrated to be of high significance. For example, implantation of only 100 ovarian cells with $CD44^+$/$CD117^+$ phenotype could develop tumors; while inoculation of even 1×10^5 CD44-/CD117- ovarian cells were not tumorigenic in athymic BALB/c nude mice. $ALDH1A1^+$ cancer cells display higher *in vitro* tumorigenecity, whereas isogenic $ALDH1A1^-$ cells do not have similar effects. Similarly, side population of pancreatic cancer cells enriched with $CD24^+$/CD44+/CD133+ demonstrate increased tumorigenecity upon xenografting in nude mice and enhanced chemoresistance against gemcitabine. Certain transcription factors and signaling pathways are critically involved in the self-renewal, differentiation and maintenance of cancer stemness; these include Oct4, Sox2, Nanog, KLF4 genes, and Notch, Hedgehog, and Wnt pathways. For example, the Sox2, Nanog, and other stemness associated transcription factors are responsible for maintaining CSC phenotypes such as self-renewal capacity, evasion from apoptosis, enhanced tumorigenesis, drug-resistance, and metastasis-associated properties in

multiple malignancies. Moreover, inhibition of signaling pathways such as hedgehog and mTOR, decreased self-renewal and chemo-resistance through the downregulation of stemness associated transcription factors like, Oct4, Nanog, Sox2, KLF4, and Gli. Considering the significance of CSCs in various malignancies, there is a clear need to eliminate the CSC subpopulation in order to achieve therapeutic benefits. So far several studies have successfully demonstrated that exploiting the antibodies against CSC specific surface markers, inhibition of signaling pathways regulating CSCs and utilizing immunotherapy could result in the inhibition of CSCs, thereby decreasing the tumor development, metastasis, and chemoresistance. For targeting CSCs, several challenges exist, namely identification of CSCs may be difficult because many cell surface markers like CD44 or CD24, may not be present on all CSCs; putative targets for CSCs like phenotypic surface markers, microenvironment and signaling pathways are also essential for normal stem cells and thus, may not represent true and specific CSCs target. Several potential therapies that target the CSC population while sparing the normal stem cell population are being explored in neuroblastoma. Further studies are highly warranted to identify the CSCs with high specificity and devise the strategies that could specifically target the CSC population.

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21

Stem Cell Signaling in the Heterogeneous Development of Medulloblastoma

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21.1 Brain Tumor Cancer Stem Cells

Brain and central nervous system (CNS) tumors are the most common neoplasms for ages 0–19 years (5.42 per 100,000), and the seventh most common in adults (27.86 per 100,000) (Ostrom et al., 2014). There are over 120 varieties of brain tumor that may be either benign or malignant. While relatively rare, the mortality rate of brain tumors is high compared to other cancers due to limitations of current treatment options and the nature of the disease. At present, brain tumors are responsible for the highest rate of cancer-related death in children.

There is a great deal of biological diversity found in brain tumors. This can be observed in the comparison of individual cases, as well as in the complexity of cellular populations that make up a single tumor. For example, the presence of undifferentiated subpopulations in adult and infant tissues that is responsible for tissue maintenance and development. Neural stem cells were first identified in mouse models, which led to the discovery and isolation of human neural stem cell populations (Reynolds and Weiss, 1992; Roy et al., 2000). Understanding the characteristics of stem cell populations is of upmost importance for cancer research due to the discovery of cancer stem cells (CSCs). CSCs are a small subset of cells that are capable of unlimited self-renewal, can undergo differentiation, and are tumor-initiating when transplanted orthotopically (Figure 21.1A). The general premise of the "cancer stem cell hypothesis" is that not all cells have

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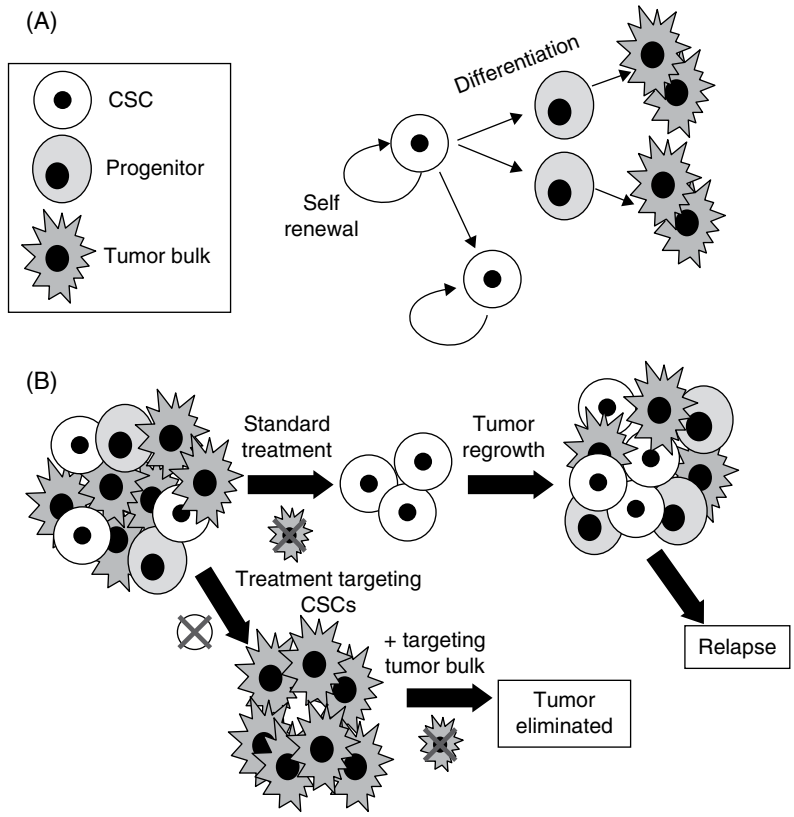

Figure 21.1 Summary graphic of the cancer stem cell hypothesis. *(A) Cancer stem cells (CSCs) have the ability to self-renew and differentiate into tumor bulk to re-establish tumor formation upon relapse. (B) Standard treatments that fail to target CSCs result in tumor regrowth, whereas elimination of CSCs in combination with therapies that target the growth of more differentiated cells may prevent relapse*

equal ability to proliferate and maintain tumorigenesis. While the bulk of the tumor is made up of differentiated cells, the CSC population can continually self-renew and asymmetrically divide to generate tumor mass. It is hypothesized that cancer treatments fail to prevent tumor relapse as they target the tumor bulk and not CSCs (Tan et al., 2006; Vescovi et al., 2006). The neurosphere assay is a convenient means to assess changes in self-renewal *in vitro*. It is particularly useful when assessing novel drugs that might inhibit CSC but not normal human neural stem cells (hNSCs).

Singh and colleagues were the first to isolate and characterize brain tumor CSCs from the malignant brain tumors, medulloblastoma (MB) and glioblastoma (GBM) (Singh et al., 2003). *In vitro*, brain CSCs are grown and propagated in selective neurosphere culture conditions that are non-adherent, and in serum-free medium supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF). A neurosphere is a "ball of cells" generated from the self-renewal and proliferation of viable CSCs. Some semi-differentiated progenitor cells can also generate neurospheres, but the true test of CSC function is whether the cell population can sustain neurosphere growth following multiple serial passages (Singec et al., 2006). Extraction of primary brain tumor neurospheres is a valuable practice because primary neurospheres will often more closely retain the characteristics of the neoplasm of origin when compared to cell lines that are propagated adherently in serum (Lee et al., 2006).

Prominin 1 (CD133) is a cell surface glycoprotein suggested to be a marker of multipotent stem cells in the brain and other tissues (Galli et al., 2004; Singh et al., 2003). CD133-positivity correlates with the CSC population and is enriched for in the subventribular zone leading to the question of whether these cells are the cells of origin for malignant glioma. When sorted using flow cytometry and orthotopically transplanted into immunocompromised mice, CD133-negative cells require $>10^5$ cells for tumor initiation whereas the CD133-positive population could generate tumors with as few as 100 cells (Singh et al., 2004). GBM having >2% CD133-positive cells or high Ki67 staining are considered to have a poor prognosis (Pallini et al., 2008).

CD15 (stage-specific embryonic antigen 1/SSEA-1/FUT4) is another proposed marker of brain tumor CSCs (Read et al., 2009; Son et al., 2009). Sorting for CD15-positive cells enriches for tumor initiation and proliferation; however, there is a lack evidence of multi-lineage differentiation. As well, CD15-positive MB cells do not consistently form self-renewing neurospheres and are expressed in lineage-committed GNP cells (Read et al., 2009).

Aldehyde dehydrogenase (ALDH) is a family of metabolic enzymes that catalyze the oxidation of aldehydes, which are toxic products of alcohol metabolism (Lipsky et al., 2001). A relationship between high ALDH activity and stem cell behavior prompted the use of an ALDH based fluorescence assay, Aldefluor®, to identify undifferentiated populations both within cancer and normal tissues (Corti et al., 2006; Deleyrolle et al., 2011; Rasper et al., 2010). In cancer studies, high ALDH expressing cells have been associated with enhanced xenograft tumor formation in mice and chemotherapeutic resistance (Choi et al., 2014; Schäfer et al., 2012).

An exact marker for, and the origin of CSCs remains a controversial topic. The overlap between developmental pathways and drivers of tumorigenesis causes one to postulate how traits within lineage hierarchies influence brain tumor formation. Experimental evidence supports the idea that glioma CSCs could arise from abnormal stem cells that gain oncogenic traits, and also supports the possibility that they could form due to the activation of stem cell genes in more restricted progenitors (Alcantara Hambardzumyan et al., 2009; Llaguno et al., 2009; Read et al., 2009). While it was initially thought that CD133-negative cells represented a non-tumor initiating population, it was later shown that they can generate tumors that also contain CD133-positive cells (Wang et al., 2008). This plasticity may suggest that CD133 is not a reliable marker of CSCs; in addition, CD133 is also expressed by endothelial cells that may contaminate the sorting of GBM cells, skewing experimental results. Differing traits between CD133-positive and CD133-negative cells might even represent CSCs from distinct cells of origin. Gene expression profiles suggest CD133-positive cells resemble a proneural glioma signature while CD133-negative correlates with a mesenchymal signature (Lottaz et al., 2010).

Regardless of markers, resistance to chemotherapy and radiation regimes is the most important characteristic of CSCs. While treatments often are successful in killing off the tumor bulk, CSCs have unique properties that allow them to continue to proliferate and self-renew thereby regrowing the neoplasm (Figure 21.1B) (Diehn et al., 2009; Liu et al., 2006; Stiles and Rowitch, 2008). Radioresistance can result from the activation of DNA damage response pathways (Bao et al., 2006; Hambardzumyan et al., 2008). As well, CSC populations have been shown to elevate expression of anti-apoptotic and drug resistance proteins such as MGMT. This increase results in heightened resistance to chemotherapies, namely carboplatin, etoposide, paclitaxel and temozolomide (TMZ) (Liu et al., 2006). It is absolutely critical that new therapies be developed to eliminate the tumor bulk, while also targeting CSC subpopulations, in order to prevent relapse of high-grade brain tumors.

21.2 Medulloblastoma

Medulloblastoma (MB) is the most frequently occurring malignant brain tumor in children that accounts for upwards of 20% of pediatric brain tumors (Crawford et al., 2007). Twenty-one percent of MB occurs in infants with the peak incidence of approximately 44% MB of pediatric MB occurring between the ages of

4–9 years (Kool et al., 2012; Tabori et al., 2006). Improvements to treatment regimes have dramatically increased progression-free and overall survival rates, but unfortunately, for many MB patients, standard radiotherapy and surgical procedures still fail to prevent tumor regrowth and cancer relapse. The discovery of CSC populations has offered some explanation into the complexity of recurrent MB. Researchers are now searching for clinically approved drugs that can be repurposed for the elimination of therapy-resistant tumor subpopulations.

21.3 Hijacking Cerebellar Development

Tumor formation is often the result of aberrant signaling pathways that are normally required for the growth and maintenance of specific tissues. As an embryonic tumor, strong evidence suggests that specific stem cell signaling pathways are responsible for MB formation. To strategically develop therapy and prognostic markers, an understanding of how stem cell pathways drive MB biology is essential.

21.3.1 Cerebellum Development

The cerebellum has a number of different precursor cell populations that are regulated by signaling pathways during development. There are two major embryonic germinal zones from which cerebellar cells develop. The first is the ventricular zone (VZ) that lines the roof of the fourth ventricle. It contains GABAergic precursor cells that will later form cell types such as Purkinje neurons, astrocytes, oligodendrocytes and Bergman glia (Eberhart et al., 2008). The second cerebellum germinal zone is the rhombic lip. This is the location of glutamatergic precursors that migrate to the nuclear transitory zone to form the deep nuclei. In addition, the rhombic lip produces granule neuron precursor (GNP) cells that proliferate transiently to form the external granular layer (EGL). GNPs are considered lineage-committed and will differentiate into mature granule neurons that form an internal granule layer (IGL). As well, GNPs express high levels of the transcription factor *ATOH1* (*MATH1*), which is essential for EGL development and used as a marker for GNP cells. It has been suggested that another population of undifferentiated stem cells may exist within the white matter of the postnatal cerebellum. It is interesting to note that this population can generate astrocytes, neurons, and oligodendrocytes, but does not generate GNPs (Goldowitz and Hamre, 1998; Pei et al., 2012a; Wang and Zoghbi, 2001). As this variety of embryonic precursor cells develops into a postnatal cerebellum, there are key signaling pathways that control this process.

21.3.2 WNT Signaling

The WNT pathway was originally discovered through the *wingless* phenotype of *Drosophila melanogaster* developmental studies. In humans, it has an important developmental role in the embryonic brain and neural stem cell signaling (Ciani and Salinas, 2005). Pei and colleagues (2012a) have shown that WNT signaling promotes the proliferation of GABAergic neural stem cells located in the VZ. It is suggested that elevated WNT signaling not only drives proliferation but disrupts neural stem cell differentiation (Pei et al., 2012a). The activity of the WNT pathway depends on the cellular accumulation of free β–catenin (CTNNB1). Normally maintained at low levels, β–catenin is contained by an APC/AXIN/GSK3 multiprotein complex. GSK3 or another kinase, CK1α, will phosphorylate β–catenin and thereby target it for ubiquitination and proteolysis. The canonical pathway is activated when extracellular WNT ligands bind frizzled (FZD), a family of G-protein coupled transmembrane receptors. Activation of FZD sends a direct signal to the cytosolic protein disheveled (DSH) that causes a disruption of the inhibitory APC/AXIN/ β–catenin complex. This liberates β–catenin from targeted degradation and it increases cytosolic concentration. β–catenin will translocate

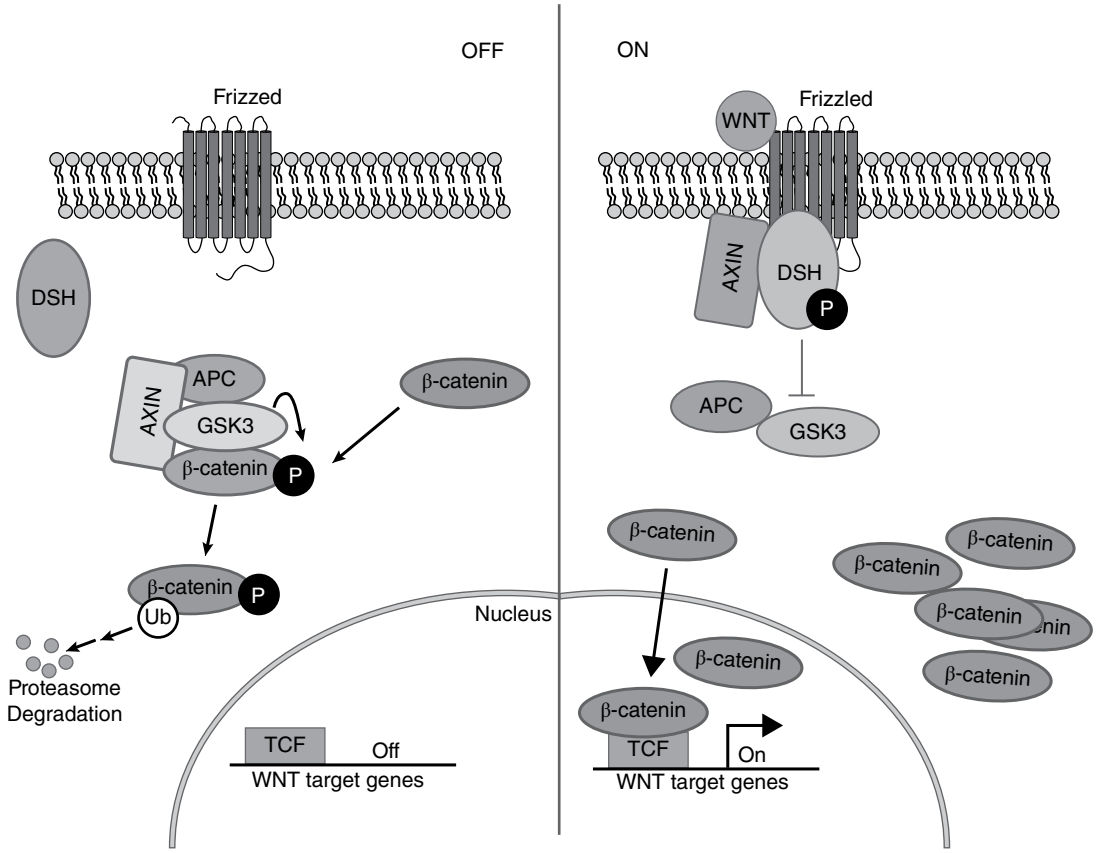

Figure 21.2 *Summary graphic of canonical WNT signaling pathway*

to the nucleus where it interacts with TCF co-factor proteins to activate transcription of WNT pathway genes such as Cyclin D1 and c-MYC (Figure 21.2). Activation of WNT pathway transcript substrates affects proliferation, and cell fate determination (Zechner et al., 2003).

Familial activating mutations in the WNT pathway have been reported to cause MB. Specifically, Turcot's syndrome results from the mutation of the adenomatous polyposis coli (*APC*) gene and predisposes for MB and GBM (Thompson et al., 2006). Modifications to APC affect its ability to complex with β–catenin thereby blocking regular β–catenin degradation programs. Somatic mutations can also occur in the gene that codes for β–catenin, *CTNNB1*. This mutation disrupts the negative regulatory systems that tightly control β–catenin resulting in accumulation in the nucleus (Eberhart et al., 2000; Zurawel et al., 1998).

21.3.3 Sonic Hedgehog (SHH) Signaling

SHH is one of three human homologs of the *Drosophila melanogaster* Hedgehog (*Hh*) gene, and its expression is crucial to neural tube and brain development early in embryogenesis (Odent et al., 1999). GNPs, after migrating to the EGL, proliferate in response to SHH ligands that are secreted by Purkinje neurons. In the canonical pathway, SHH ligands bind the transmembrane receptor patched (PTCH1) on the surface of GNPs. When not bound by SHH, PTCH1 represses the activity of another transmembrane protein, smoothened

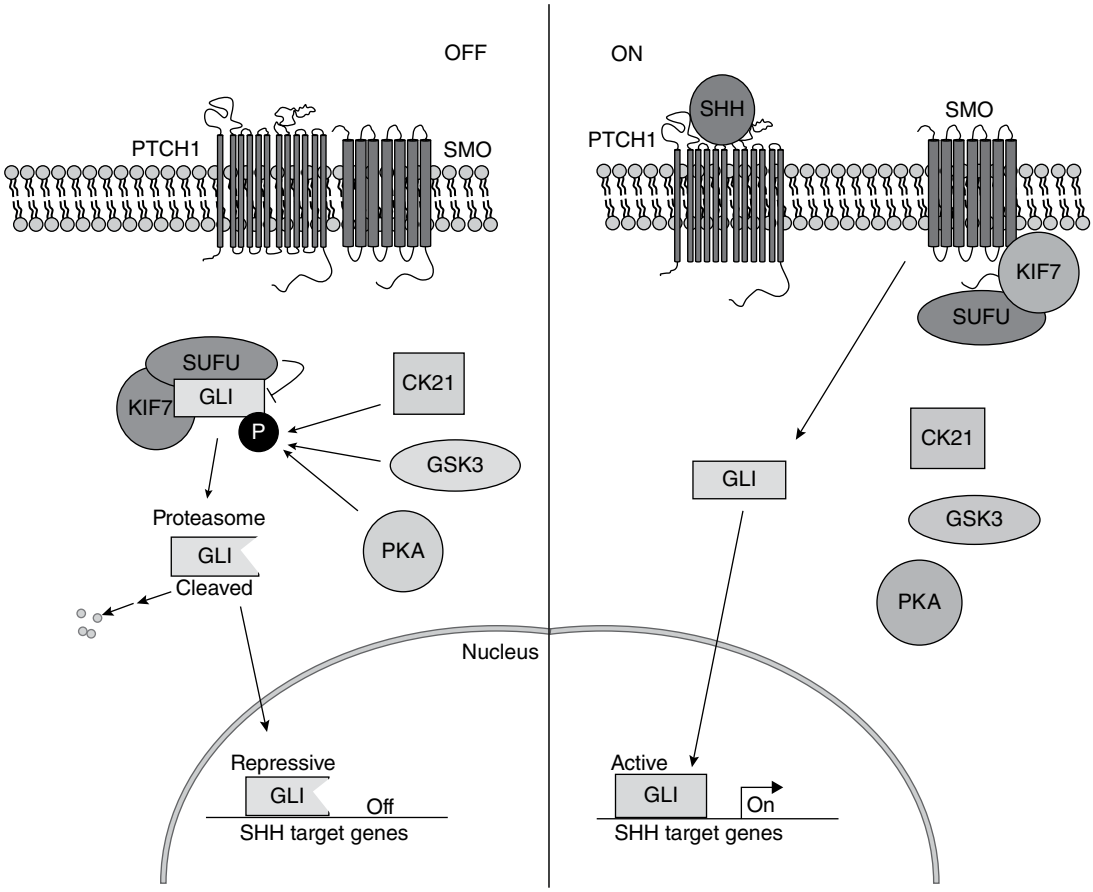

Figure 21.3 *Summary graphic of canonical SHH signaling pathway*

(SMO). Ligand binding results in the internalization of PTCH1 thereby releasing the inhibition of SMO that then recruits and binds other pathway components to the membrane. Within the cell, the proteins SUFU and Kif7 sequester a pool of microtubule-bound GLI transcription factors. PKA, CK21, and GSK3 are kinases that oppose the SHH pathway by phosphorylating GLI when sequestered; this causes GLI degradation (Ingham et al., 2011). Proteasome-dependent cleavage of GLI also results in the conversion of carboxy-terminus repressor GLI proteins that are able to translocate to the nucleus and block the transcription of SHH target genes (Ruiz et al., 2002).When SMO is active, the repression of GLI is released, and it translocates to the nucleus. Nuclear GLI binds DNA to activate or repress specific genes such as *PTCH1*, *HHIP*, *MYCN*, *BMI1*, and *ATOH1* (Dahmane et al., 1997; Ruiz et al., 2002) (Figure 21.3).

Mutations found in the gene coding for PTCH1 are responsible for causing Gorlin's Syndrome, a disease that increases susceptibility for MB (Cowan et al., 1997). This autosomal dominant disorder is also associated with mental defects. Other mutations affecting *SMO, SUFU,* and *GLI1/2* may cause constitutive activation of the SHH pathway, thereby promoting dysregulated proliferation in the GNP cell population that can result in MB tumor formation (Schüller et al., 2008; Taylor et al., 2002). Along with embryonic development, the SHH pathway regulates neural stem cells in the adult brain; however, this regulation in non-GNPs is not as well characterized (Palma et al., 2005). Notably, crosstalk of the SHH pathway with other signaling networks – such

as WNT, BMP, NOTCH, and MYC – is in part responsible for controlling both normal and cancer cell development (Natarajan et al., 2013; Oliver et al., 2003; Pan et al., 2006; Rios et al., 2004).

21.3.4 BMP Signaling

Members of the bone morphogenic protein (BMP) family are thought to be responsible for providing the initiatory signal for granule cell specification of precursors in the rhombic lip. BMPs – specifically BMP6, BMP7, and GDF7 – are produced by cells in the dorsal midline (Alder et al., 1999). This pathway stimulates GNPs to begin to differentiate into mature granule neurons that eventually form the IGL. BMP2 signaling is reported to suppress the proliferative response of GNPs to SHH to cause cell cycle exit (Angley et al., 2003; Rios et al., 2004; Zhao et al., 2008). Alder and colleagues demonstrated that treating neural cells with BMPs and transplanting them into the postnatal mouse cerebellum causes the differentiation of mature granule neurons in the EGL (Alder et al., 1999). As a negative regulator of SHH-driven cell proliferation in the cerebellum, BMP signaling is highlighted as an influential regulation pathway in MB (Fogarty et al., 2005; Merve et al., 2014).

21.3.5 NOTCH Signaling

The proliferation and differentiation of GNPs are also coordinated by the NOTCH pathway (Yoon and Gaiano, 2005). This pathway is characteristically involved with cell-cell communication, and its canonical pathway includes juxtacrine binding of membrane-bound ligand receptors (Natarajan et al., 2013). The transmembrane receptor, Notch2, binds a membrane-bond ligand (i.e., JAG1/2 or DLL family) from another cell to activate cleavage of its intracellular domain, NICD, which then translocates to the nucleus to activate transcript of genes like p21, MYC and the HES family. There are multiple proteins that modulate this pathway (Solecki et al., 2001; Yoon and Gaiano, 2005). *NOTCH2* signaling is down-regulated as GNPs exit the cell cycle. The NOTCH pathway reportedly inhibits differentiation and promotes proliferation, which has implications for sustaining oncogenesis if dysregulated (Solecki et al., 2001). Hallahan and colleagues have shown that *NOTCH2* and *HES5* pathway members are elevated in MB compared to normal cerebellum; as well, they have been linked to SHH signaling using *in vivo* models of MB (Hallahan et al., 2004). An understanding of the biology of development is an asset in decoding the biology of cancer.

21.4 Molecular Classification of MB

The classification of molecularly distinct subgroups within single cancer types has become common in oncology. Genomic technologies and advanced biostatistics have identified four subgroups of MB based on patient demographics, clinical outcomes, somatic genetic events, and gene expression (Taylor et al., 2012). The establishment of these subgroups is a result of over a decade of research that depicts the influence of specific cell signaling pathways and identified chromosomal abnormalities. The initial attempts to deduce the clear number of subgroups by different research groups had variable results, mainly due to variation in patient cohorts (Cho et al., 2011; Kool et al., 2008; Northcott et al., 2011a; Thompson et al., 2006). Cooperation within the research community resulted in the acceptance of four main subgroups at a consensus meeting held in 2010 (Taylor et al., 2012). These include: WNT, SHH, Group 3, and Group 4 (Table 21.1).

Northcott and colleagues (2011b; 2012c) have created a NanoString based molecular subtyping method for MB. A 25-gene minimal marker signature can be used to determine subtype using low quantities of RNA extracted from FFPE. In an early study researchers were able to assign either WNT, SHH, Group 3, or Group 4 classification to 88% of clinical FFPE samples assessed with 100% accuracy (Northcott et al., 2012c).

Table 21.1 *General summary of MB molecular subtype characteristics*

	WNT	SHH	Group 3	Group 4
Proportion of cases:	~10%	20–25%	25–30%	35–40%
Common age groups:	Children, few adults	Adults and infants, some children	Young children, some infants	Mostly children, few infants and adults
Gender ratio (F/M):	1:1	1:1.5	1:2	1:3
Prognosis:	Good	Intermediate to Poor	Poor	Intermediate
Proposed cell of origin:	Lower rhombic lip and dorsal brainstem progenitor cells	-Neural stem cells from SVZ -GNPs from EGL	-Neural stem cells -GNPs from EGL	Unknown
Signaling Expression:	WNT pathway	SHH pathway	-MYC signature -Retinal genes	Neuronal genes
Histology:	Mostly Classic	DN, Classic, LCA, MBEN	Classic, LCA	Classic, LCA
Cytogenetics	Monosomy 6	$3q^+$, $9q^-$, $10q^-$	i17q, $1q^+$, 7^+, $10q^-$, $16q^-$, $17q^-$	i17q, X^-, $11p^-$, 7^+, $18q^+$
Examples of altered genes:	*CTNNB1, APC, SMARCA4, CREBBP, DDX3X, PIK3CA*	*PTCHI, SMO, SUFU, TP53, MYCN, GLI2*	*MYC, OTX2, SMARCA4, KMT2D, CHD7*	*MYCN, CDK6, SNCATP, KDM6A, MLL3, ZMYM3, CBFA2T2*

Adapted from (Northcott et al., 2012a, 2012b; Taylor et al., 2011)

The reason some samples could not be subtyped is attributed to the age and degradation of archived FFPE tumor tissue. NanoString nCounter technology offers an opportunity to predict MB tumor subtype that may be instrumental in providing accurate patient stratification and dictate effective treatment protocols.

21.4.1 WNT Subtype

The WNT subtype of MB is the most established but occurs in fewer than 10% of cases (Northcott et al., 2012a). These cases are generally older children and lack leptomeningeal dissemination. Over 90% of individuals with WNT MB have long-term survival, and this subtype is considered a good prognosis. Ellison and colleagues report that the deaths that do occur in the WNT subgroup are usually due to complications of treatment of due to the formation of a secondary neoplasm (Ellison et al., 2011). Interestingly, LCA histology has been reported in a few rare WNT cases and is considered to have a good prognosis (Ellison et al., 2005), however, the vast majority of WNT MB have CMB histology. Traditional clinical stratification methods are useless in identifying these cases without additional markers.

As the name suggests, tumors in the WNT subgroup are recognized for a hyper- activation of the WNT, or β–catenin, signaling pathway. Familial activating mutations in the WNT pathway have been reported to cause MB. Specifically, Turcot's syndrome results from the mutation of the *APC* gene and predisposes for MB and GBM (Thompson et al., 2006). Modifications to APC affect its ability to complex with β–catenin thereby blocking regular β–catenin degradation programs. As a result of the loss of negative β–catenin regulation,

it continues to translocate to the nucleus and activate the WNT signaling transcriptome. Somatic mutations can also occur in *CTNNB1*, and this mutation disrupts the negative regulatory systems that tightly control β–catenin resulting in accumulation in the nucleus.

Monosomy of chromosome 6 is the main cytogenetic characteristic of the WNT subtype (Ellison et al., 2011), and more recently elevated expression of CD15 (Manoranjan et al., 2013). Mutations in SMARCA4, CREBBP, DDX3X, and PIK3CA have also been found in WNT subgroup tumors (Robinson et al., 2012). In addition, immunohistochemical staining for high nuclear β–catenin or DKK1 positivity are useful tools for identifying WNT cases that has already been adopted by some pathologists (Ellison et al., 2005; Northcott et al., 2011b).

21.4.2 Sonic Hedgehog (SHH) Subtype

Between 20–25% of MB are classified in the SHH subtype (Northcott et al., 2012a). This group was originally considered to have an intermediate to poor prognosis (Kool et al., 2008; Pietsch et al., 2014). There may exist further subcategories of SHH MB that accounts for its clinical heterogeneity (Kool et al., 2008; Pietsch et al., 2014). Between 15–20% of cases diagnosed present metastatic disease and there is an interesting bimodal distribution in patient age. (Northcott et al., 2012b, 2011b). Compared to other subtypes, there is a higher frequency in patients younger than 3 (infant) and older than 16. Clear differences in molecular and clinical attributes of pediatric and adult SHH MB have previously been reported (Northcott et al., 2011a). Histologically there is an enrichment of desmoplastic nodular (DN) cases in the SHH subtype, however, while nearly all DN tumors are SHH, SHH tumors can also have CMB, LCA, and MBEN histology (Ellison et al., 2011). To add further complexity, having DN histology is suggested to be a good marker of prognosis for younger children, but a poor prognostic marker for adult cases (Northcott et al., 2011a).

The SHH subtype is another group identified based on the dysregulation of a previously studied signaling pathway. Compared to other MB subtypes, the highest rates of single gene mutations occur in the SHH subtype (Northcott et al., 2012b). Somatic mutations in *PTCH1*, *SUFU*, and *SMO,* as well as amplifications of *GLI1* and *GLI2,* have been found in MB (Kool et al., 2014; Parsons et al., 2011; Pugh et al., 2012; Taylor et al., 2002). There is also a number of SHH cases that have MYCN amplification. Both Group 4 and SHH groups can have MYCN overexpression whereas Group 3 MB are known for overexpression of a relative protein, c-MYC (Kool et al., 2008; Northcott et al., 2011b). SHH-driven MB also has identifying chromosomal aberrations, for example, the deletion of 10q, 14q, or 17q, and gain of 3p or 3q. Interestingly, *PTCH1* is located at 9q22, and the loss of 9q has also been reported in a small number of tumors. (Northcott et al., 2012b). Northcott and colleagues proposed the use of secreted frizzled-related protein 1 (SFRP1) protein to identify SHH cases, however, immunohistochemical detection of SHH has been less reliable than gene expression based methods (Northcott et al., 2011b).

21.4.3 Group 3 Subtype

Group 3 MB are considered to have the worst prognosis of the four subtypes. This group is diagnosed in ~27% of MB and is associated with the highest rates of metastatic disease that are reported to be between 40–45% of patients (Kool et al., 2012; Taylor et al., 2012). The gender distribution is skewed with a 2:1 male to female cases and Group 3 occurs almost exclusively in pediatric cases. Originally no adult Group 3 MB were reported (Remke et al., 2011), but larger meta-analysis suggest they account for 6% of this group (Kool et al., 2012). This discrepancy can most likely be attributed to the difference in age cut-off used in these studies, as the classification of young adults (16–21) can be variable. Regardless, Group 3 MB is especially aggressive with an overall survival of only 33% adult, 39% infant, and 50% childhood cases. Agreeing with these survival trends, LCA histology occurs more frequently in Group 3 MB. CMB histology also occurs in Group 3 whereas DN and MBEN are absent from this subtype (Kool et al., 2012; Northcott et al., 2012a, 2011b).

Unlike WNT and SHH subtypes, Group 3 and Group 4 have more similar genetic profiles that rely on gene expression-based diagnosis. A single signaling pathway has not yet been identified as being responsible for Group 3 oncogenesis; however, there is precedence for overexpression of genes associated with retinal development. Orthodenticle Homeobox 2 (*OTX2)* is one such gene that is amplified in 7.7% of Group 3 MB (Adamson et al., 2009; Bai et al., 2010; Northcott et al., 2012b). The most highly associated genomic aberration is *MYC* amplification (16.7%), which occurs mutually exclusive to *OTX2* amplification. *MYC* overexpression is a candidate driver of this subtype and a marker of poor prognosis (Delmore et al., 2011; Northcott et al., 2012b, 2011b). It has also been suggested that Group 3 can be stratified into two subsets, Group 3α, which has amplified *MYC* and poor prognosis, and Group 3β, which does not have the amplification and experiences an intermediate prognosis (Cho et al., 2011). Although fluorescence *in situ* hybridization (FISH) detection of MYC is a commonly used in laboratories, there are limitations to sensitivity using this assay (Pfister et al., 2009). Similarly, natriuretic peptide receptor 3 (NPR3) has been developed as an immunohistochemical marker for the diagnosis of Group 3 MB, but its detection lacks the sensitivity required for robust clinical use (Min et al., 2013; Northcott et al., 2011b).

More recently, mutations affecting epigenetic regulatory proteins were discovered in Group 3 MB. For example, *SMARCA4*, *KMT2D*, and *CHD7* encode for proteins that remodel chromatin, as well as mutations affecting genes in the lysine-specific demethylase (KDM) family (Jones et al., 2013, 2012; Northcott et al., 2012a). More work is currently underway to decipher which important downstream systems are modified by these epigenetic signaling mutations. Single gene mutations are more common in SHH subgroup and are found in less than half Group 3 MB where chromosomal alterations are more prevalent. A gain of chromosome 1q, 7, 17q, and 18, as well as loss of 10q, 16q, and 17q have all been reported (Northcott et al., 2012b; Pfister et al., 2009). Researchers are now beginning to pinpoint functional reasons as to why such chromosomal alterations drive MB progression. In a recent publication, somatic genomic rearrangements were found to increase the expression of *GFI1* and *GFI1B* in one-third of Group 3 MB. It is suggested that rearrangements of chromosomal DNA modifies the proximity of enhancer regions to the promoters of these oncogenes thereby elevating expression within the cell (Northcott et al., 2014). Under this premise, the downregulation of genes as a result of chromosomal rearrangements is also a consideration that requires further investigation.

21.4.4 Group 4 Subtype

The final and most common occurring MB subgroup is Group 4. Just over 35% of MB are classified as Group 4 and between 35–40% of these cases are metastatic at the time of diagnosis (Kool et al., 2012; Northcott et al., 2012a). The highest disproportion in gender distribution is within this group, as there are three boys diagnosed for every one girl (Kool et al., 2012; Northcott et al., 2012a). Taylor and colleagues (2011) describe Group 4 as being "prototypical MB" in clinical appearance, however, at this point it can only be identified using transcriptional profiling. Between 40–45% Group 4 MB occur in the childhood age bracket, with 25% in adult and considered rare in infants. Group 4 has an intermediate prognosis for childhood cases but is a marker of poor outcome for adults. Infants with Group 4 MB have the worst outcome relative to other age groups (Kool et al., 2012; Remke et al., 2011; Shih et al., 2014). Histologically, the majority of Group 4 patients have CMB with a few cases of LCA. DN has been reported in Group 4, but it has been suggested that this may have been due to a categorization error (Gajjar and Robinson, 2014; Kool et al., 2012). Further, LCA and Group 4 tumors with metastasis are considered high risk and show about 60% 5-year survival. Standard risk Group 4 cases with no metastasis and gross total resection have >80% 5-year survival with current treatment strategies. (Kool et al., 2012; Shih et al., 2014; Taylor et al., 2012). Recent studies using cytogenetic markers demonstrate a low-risk population of Group 4 based on chromosome 11 loss or 17 gain. Both low and standard risk Group 4 individuals have the potential to do well with current treatment strategies (Shih et al., 2014).

Although Group 4 cases occur frequently, there is least known about the molecular pathology of this subgroup. There are no known syndromes that predispose for Group 4 MB, and it was originally identified based on the bioinformatic clustering of cases that overexpress neuronal differentiation and glutamatergic genes (Kool et al., 2008; Northcott et al., 2011b). Aside from gene expression, the use of potassium voltage-gated channel subfamily A member 1 (KCNA1) has been proposed as an immunohistochemistry (IHC) marker of Group 4, however, validation studies found it to be detectable in all four subgroups making it ineffective for classification (Min et al., 2013; Northcott et al., 2011b). The most common aberration in Group 4 is an isochromosome 17q (i17q), which is found in 66% of cases. This aberration occurs when an additional q-arm is used to replace a lost p-arm of chromosome 17 (Kool et al., 2012; Northcott et al., 2012a; Robinson et al., 2012). Although never seen in WNT or SHH tumors, i17q is present in both Group 3 and Group 4 MB. Of note, i17q is a marker of poor prognosis in Group 3 where it is found in 26% of cases, but has no prognostic influence in Group 4 relative to balanced karyotype patients (Shih et al., 2014). Other rearrangements that have been found in Group 4 include gains in chromosome 7, 17q, 18q, and loss in 11p, 8, and X. Approximately 80% of Group 4 female patients have a complete loss of X chromosome (Kool et al., 2008; Northcott et al., 2011b; Taylor et al., 2011).

One of the initial challenges is finding characteristics that differentiate Group 4 from Group 3 tumors. For example, *MYCN* and *CDK6* are amplified in approximately 6% and 5% of Group 4 MB, respectively, but rarely found in Group 3 (Cho et al., 2011; Northcott et al., 2011b). *SNCATP,* a gene implicated in Parkinson disease, is also amplified in around 10% of Group 4 tumors. The mechanistic importance of this modification in MB is still to be determined (Northcott et al., 2012a, 2012c). Other genes that are mutated in Group 4 include *KDM6A* (12%), *MLL3* (5%), *ZMYM3* (5%), and *CBFA2T2* (3%) (Cho et al., 2011; Northcott et al., 2012b; Robinson et al., 2012). Like Group 3 MB, amplification of OTX2, overexpression of GFI1, and mutations in the KDM protein family are routinely found. However, unlike Group 3, there is a greater proportion of amplified MYCN than MYC in Group 4 (Gajjar and Robinson, 2014; Jones et al., 2012; Northcott et al., 2012b).

21.5 Mouse Models and Cell of Origin

Model for WNT subtype of MB. Gibson and colleagues (2010) developed the first WNT specific animal model. This was achieved through the induction of *CTNNB1* activating mutations in progenitor cells of the lower rhombic lip and embryonic dorsal brainstem. Herein the first evidence of a different cell of origin between subtypes was established (Gibson et al., 2010). The progenitor cell populations driven by WNT signaling appear to differ in location and activity compared to SHH-driven progenitors. This same group later improved their first WNT model, which had 15% penetrance, to add a *PIK3CA*^{E545K} mutant allele. This mutant had been identified in human WNT tumors, and its addition increased the WNT mouse model to 100% penetrance (Robinson et al., 2012). In support of the first model, an independent group found that the Gibson and colleagues (2010) mouse model accurately resembled human tumors using gene expression profiling (Pöschl et al., 2014). The creation of animal models that truly replicate the characteristics of WNT tumors is essential for creating better methods to safely eliminate tumor growth in children.

Model for SHH subtype of MB. Mouse models of the SHH subgroup of MB have fueled much of the information known about MB. There are more models of SHH than any of the other subtypes, and they are mostly derived through the modification of various components of the SHH pathway. For example, the first models were generated using germline deletion of one *Ptch1* allele (*Ptch1*$^{+/-}$) (Goodrich et al., 1997; Wetmore et al., 2000). Similarly, another group has developed a transgenic mouse model using *Smoothened* (*Smo*) mutations (Hallahan et al., 2004; Hatton et al., 2008). These animal studies have revealed the cell of origin for SHH MB

through the promotion of tumorigenesis with deletion of *Ptch1* specifically in committed cerebellar GNPs (Schüller et al., 2008; Yang et al., 2008). More recently, genomic studies have enhanced the $Ptch^{+/-}$ penetrance with the addition of the *Sleeping Beauty* (SB) transposon system to study metastatic dissemination and screen for gene candidates responsible for driving tumorigenesis (Dupuy et al., 2005; Genovesi et al., 2013; Wu et al., 2012). The development of animal models of the SHH has been a valuable tool for understanding this MB subtype.

Model for the Group 3 subtype of MB. Less is known about the molecular drivers of Group 3 making the generation of animal models more difficult compared to the WNT or SHH subtypes. In the same journal issue, two groups are credited with developing the first animal models of Group 3 using orthotopic transplantation of murine neural progenitor cells. Pei and colleagues (2012b) did so using orthotopic transplantation of cerebellar stems cells, which were modified to overexpress *Myc* (*MYC*) along with mutant *Trp53* (p53). The tumors that developed resembled LCA histology including cell wrapping morphology and a gene expression profile that differed from SHH based mouse models (Pei, et al., 2012b). Kawauchi and colleagues (2012) also developed a *Myc* based mouse model in combination with a loss of *Trp53* in GNPs. This group used the neuronal lineage marker, *Atoh1*, to sort for GNPs, but its expression disappeared in the transformation of the tumor cells. The *Myc*-induced tumors were distinct from WNT or SHH models (Kawauchi et al., 2012). Herein, the Group 3 mouse models from both studies demonstrate that this subtype may develop from neural stem cells or de-differentiated cells as a result of MYC-dependent transformation. The aforementioned mouse models were examined for similarity to human Group 3 tumors. Although initially distinct from WNT and SHH murine models using a 50–100 gene panel, neither model matched the profile of Group 3 patient tumors; instead they were found to be most similar to SHH (Pöschl et al., 2014). The authors suggest this may be attributable to the use of loss of function p53 in the Group 3 models because this aberration is more commonly found in SHH patients. It is also valuable to note that only a portion of human Group 3 MB have MYC amplification, and more work needs to be done to establish models for other subsets of Group 3. Finally, a third research group used a patient-derived metastatic Group 3 sample to derive the cell line HD-MB03. They suggest that orthotopic transplantation of HD-MB03 into mice produced tumors reflecting the original patient tumor (Milde et al., 2012). Although patient sample xenograft may not truly reflect the developmental biology of tumor development, this approach provides useful preclinical models for therapeutic testing.

Model for the Group 4 subtype of MB. At this point in time, there are no animal models that accurately reflect Group 4 MB. This may be attributed to the current lack of understanding of the exact molecular mechanisms that drive this subgroup. Of note, the use of MYCN amplified mouse models have been suggested to partially reflect the small subset of MYCN amplified Group 4 MB (Eberhart et al., 2012; Swartling et al., 2010). As more is learned about the molecular basis of MB subgroups, greater opportunities for prognostication and therapy development have become apparent.

21.6 Additional Drivers of MB

Since the identification of the four MB subgroups, further large-scale, whole genome studies have uncovered more information that will undoubtedly refine our perception of this disease (Jones et al., 2012; Pugh et al., 2012; Robinson et al., 2012). The pathogenesis of a portion of tumors can be attributed to disruption of established development signaling pathways (i.e., WNT or SHH), however, these cases are considered a minority and the exact mechanism behind the majority of tumors is relatively unknown. One hypothesis is that the answer lies in post-translational regulation of gene expression and not solely dependent on the direct aberration of canonical pathway components.

21.6.1 Epigenetic Regulators

The role of epigenetic regulation of the oncogenome has gained a great deal of momentum in MB research. Whole-genome sequencing revealed that many of the most frequently mutated genes in MB coded for proteins with epigenetic activity. For example, mutations in *SMARCA4* and *KMT2D* are recurrently detected and not limited to specific subgroups (Pugh et al., 2012; Robinson et al., 2012). These genes are involved in chromatin remodeling. Simply, this can either increase or restrict access of transcriptional machinery to specific gene promoters. Other epigenetic factors make up the list of most frequently mutated in WNT (i.e., *CREBBP, SMARCA4, KMT2D),* SHH (i.e., *BCOR, KMT2D)*, Group 3 (i.e., *CHD7, SMARCA4, KMT2D, KDM family*), and Group 4 (i.e., *CHD7, KMT2D, KMT2C, KDM6A)* (Northcott et al., 2012a; Pugh et al., 2012; Robinson et al., 2012). Additionally, truncating mutations in *MLL2 and MLL3* also occur in a subgroup independent manner and have a significant impact on regulation of histone H3 methylation that is indicative of patient outcome (Dubuc et al., 2012).

Epigenetic factors also have influential roles within specific subtypes. BMI1 is involved in neural stem cell self-renewal and acts as a regulator in an epigenetic polycomb complex (Leung et al., 2004). Expression of *BM1I* has been linked to the SHH subtype, and there is a suggested regulatory feedback loop between BMI1 and SHH ligand in MB tumor initiating cells (Wang et al., 2011). As well, amplifications of the transcript factor OTX2 are reported in Group 3 and Group 4 MB. Bunt and colleagues suggest OTX2 activity may cause the upregulation of the epigenetic complex genes *SUZ12*, *EZH2*, and *EED* (Bunt et al., 2013; Jones et al., 2012). Experimentally silencing OTX2 caused a reduction in polycomb remodeler expression with a coinciding increase of demethylase activity. As a result this produced a decrease in histone H3 lysine 27 (H3K27) methylation, which is a key epigenetic modification suggested to allow gene expression that promotes stem cell-like properties (Bunt et al., 2013). Disruption of epigenetic regulation is a unifying theme across MB subtypes; however, the downstream consequence to transcription still remains unclear.

21.6.2 TP53

Another important driver of MB that is not restricted to a subgroup identifying signaling pathway is *TP53*. *TP53* mutations are some of the most frequently reported in cancer. This gene codes for p53, which is considered the "guardian of the genome" and has roles in controlling apoptosis, DNA repair, growth arrest, and stress response (Brosh and Rotter, 2009; Muller and Vousden, 2013; Strano et al., 2007). Li-Fraumeni syndrome is a familial disease caused by the heritable *TP53* mutations that predispose children to MB (Malkin et al., 1990). These cases normally present with LCA histology, MYCN amplification and assign to the SHH subgroup (Kool et al., 2014). Sporadic mutations of *TP53* occurs in 16% WNT and 21% SHH MB tumors (Zhukova et al., 2013). *TP53* mutations are essentially absent from Group 3, and are rare in Group 4. Patients positive for *TP53* mutation have a dramatically worse prognostic outcome (Northcott et al., 2012a; Zhukova et al., 2013). Although molecularly important, the exact genesis of *TP53* mutation in MB remains to be determined. Rausch and colleagues (2012) propose an interesting concept that links *TP53* mutations in MB to catastrophic DNA rearrangements. Specifically, they suggest that a process called chromothripsis causes a single event of dramatic chromosomal "shattering" that can result in gene mutations and copy number changes, and that this may be a driver of *TP53* mutations in SHH MB (Rausch et al., 2012). It has become clear that the pathogenesis of MB extends beyond canonical signaling pathways; therefore, our approach to treating cancer must do so as well.

21.7 Repurposing Off-Patent Drugs

One of the biggest problems in advancing the treatment of brain tumors is the time and resources required to develop new therapeutics. The presence of CSCs in brain tumors also presents a treatment challenge given they are commonly resistant to standard-of-care therapies. Perhaps identifying drug targets that define CSCs

would be a logical approach to improving treatment. For example, ALDH is a CSC gene and can also be inhibited with a drug called Antabuse or Disulfiram (DSF). This is highly desirable because the safety of DSF is well established at least in adults and it is inexpensive. It is estimated to take 15 years and somewhere between $500 million and $2 billion in preclinical and clinical testing to bring a single drug to market (Chong and Sullivan, 2007; Karamehic et al., 2013). All new drugs need to be rigorously evaluated for safety, and phase I clinical trials alone typically take 2 years and over $17 million to complete (DiMasi et al., 2003). According to one health report, only 1 in 1000 compounds in preclinical testing will proceed to clinical trials. From those, 1 in 5 will be approved for use in humans, and only 2 in 10 of those drugs approved will generate sustainable revenue to recoup development costs (Karamehic et al., 2013). As a result, the pharmaceutical industry often delegates its resources toward developing therapeutics for larger target populations and not towards less common diseases. This is especially true for pediatric cancers, which are often highly complex, and childhood specific clinical trials are exceptionally rare (Milne and Bruss, 2008).

There are nearly 10,000 drugs known to modern clinical medicine, and the full multitude of functions for the majority of these compounds is still to be determined. Currently, only 1 in 10 drugs tested clinically is covered by exclusivity patents, which leaves a plethora of drugs that could potentially be repurposed in an affordable manner. By repurposing off-patent drugs for cancer treatment, the overall cost of drug development could be cut by at least 40% due to previously conducted safety trials (Chong and Sullivan, 2007). TMZ has been the primary chemotherapeutic used to treat GBM for over a decade; this is despite the fact that most patients develop resistance to it (Stupp et al., 2005). Approximately $15,000 over an average of 5.1 months is the cost of treating a malignant glioma patient with TMZ. This consumes an estimated 61% of the total cost of patient care, and TMZ does not come close to ranking as one of the pricier cancer drugs (Meropol and Schulman, 2007; Wasserfallen et al., 2005). There is a vast amount of un-mined potential in repurposing off-patent drugs that could potentially revolutionize the treatment of high-grade brain tumors.

21.7.1 Repurposing Disulfiram (DSF)

The elevated ALDH activity of CSCs initially led researchers to test the widely known ALDH inhibitor, disulfiram (DSF), as an anti-cancer drug. DSF has been prescribed for the treatment of substance abuse since its first clinical trial in 1948 under the trademarked name Antabuse® (Hald and Jacobsen, 1948; Suh et al., 2006). Regular treatment with DSF deters patients from drinking alcohol as ALDH inhibition prevents the conversion of acetaldehyde to acetate in the liver. Consuming alcohol when taking DSF causes an increase of acetaldehyde in the body that results in the patient experiencing unpleasant flu-like symptoms. The only major negative side effect is hepatotoxicity when DSF is prescribed at high doses (Berlin, 1989).

The activity of the ALDH family of enzymes is suggested to have roles in cell detoxification and Aldefluor® active cancer cells have been associated with resistance to cisplatin, docetaxel, and doxorubicin (Jiang et al., 2009). Unfortunately, the detoxifying properties of ALDH can be counterproductive to chemotherapy treatment as the metabolism and degradation of drugs can be directly manipulated by high ALDH activity. For example, the drug cyclophosphamide is one of the most prescribed chemotherapeutics in MB treatment protocols, which has been shown to be significantly less effective in ALDH-high cells (Hipken et al., 1981; Sládek et al., 2002).

Although best characterized for its inhibition of ALDH, the full molecular effect of DSF remains to be fully understood. DSF is considered a sulfhydryl enzyme inhibitor that inhibits enzymes with disulfide bonds (Eneanya et al., 1981). This means the activity of DSF is not exclusive to ALDH, and it can function to block a number of pathways implicated in stem cell detoxification and development. DSF has been shown to reduce the activity of dopamine beta-hydroxylase (Musacchio et al., 1966), plasma cholinesterase (McCance-Katz et al., 1998), microsomal carboxylesterases (Zemaitis and Greene, 1976), cytochrome oxidases (Scheibel et al., 1979), and DNA methyltransferases (Lin et al., 2011). Adding to this, DSF also suppresses the proteasome

and NFkB pathways (Chen et al., 2006; Liu et al., 2012, 2014; Yip et al., 2011; Zha et al., 2014). This is thought to be an effect of diethyldithiocarbamate, which is a small metabolite of DSF that is formed when DSF is metabolized in the body. Diethyldithiocarbamate chelates with copper or zinc ions to form large complexes that can directly block proteasome activity, shift cellular redox, and increase oxidative stress (Yip et al., 2011). Under this premise, many cancer studies test DSF in combination with copper (Cen et al., 2002; Liu et al., 2013; Yip et al., 2011). The debate continues as to whether or not the addition of copper is important for the efficacy of DSF.

DSF was identified in multiple studies as an agent that inhibits CSCs for cancers of the blood, breast, lung, ovary, and pancreas (Hothi et al. 2012; Iljin et al. 2009; Mimeault and Batra 2008). Recently it was shown that the survival of patients with stage IV non-small cell lung cancer could be prolonged by combining DSF with current cisplatin and vinorelbine protocols (Nechushtan et al., 2015). Preclinical studies demonstrated that DSF treatment alone was sufficient to almost completely eliminate GBM (Triscott et al., 2012) and MB (unpublished) cell proliferation *in vitro*. A recent study using another brain tumor model, atypical teratoid rhabdoid tumor (AT/RT), showed that DSF treatment can reduce the CSC population and can pass through the blood-brain barrier into the brain (Choi et al., 2014). There were no negative side effects reported in the mice that were treated with DSF. Finally, an added benefit to repurposing this off-patent drug is that the cost to treat a patient for a year with DSF is estimated to be less than 1% the cost of treating the average cancer patient with current protocols (Fojo and Grady, 2009; Wasserfallen et al., 2005). Presently, two ongoing phase II clinical trials have been initiated which test DSF on GBM (ClinicalTrials.gov Identifiers NCT01907165 and NCT01777919). Pending positive outcome from these GBM trials, DSF could be repurposed for other malignant brain tumors like MB.

21.8 Emerging Therapies for MB

Patients with SHH MB are at high risk of relapse. Thus, therapies specifically aimed at disrupting that pathway have been explored, the most notable being SMO inhibitors. However, resistance to SMO inhibitors is more common than might be expected due to activation of the key kinase pathways. The MAP kinase pathway is an important driver of cancer cell growth, invasion and metastasis. It is activated by cell surface receptors as well as mutations in points along the pathway. For example, the missense mutation V600E constitutively activates B-raf. Pambid and colleagues described the importance of RSK as a point of intervention in the MAPK pathway in medulloblastomas (Pambid et al., 2014). There was clear tumor cell dependency on RSK given that silencing RSK1 and RSK2 suppressed cancer cell growth and self-renewal. Similarly, the small molecule pan-RSK inhibitor BI-D1870 suppressed growth and self-renewal. However, in normal hNSCs interfering with RSK had no effect, illustrating that inhibiting RSK uniquely eliminates cancer stem cells but spares normal hNSCs. In the normal mouse brain, RSK2 serves a role in neurogenesis where it is highly expressed at E13.5 and gradually declines to low levels by P2 (Dugani et al., 2010). It was also important to note that the RSK inhibitor BI-D1870 crossed the blood brain barrier in mice (Pambid et al., 2014).

Mitotic kinases that regulate cell duplication and division are also promising targets for MB treatment. For example, many cancer types have an oncogenic addiction to polo-kinase kinase 1 (PLK1), which is highly overexpressed in MB tumors relative to normal cerebellum (Lee et al, 2012; Pezuk et al., 2013; Takahashi et al., 2003; Triscott et al., 2013; Wolf et al., 1997). PLK1 acts as a molecular hub that both receives and transmits mitotic signals at specific times and locations that are essential for the coordination of mitosis (Archambault et al., 2015). Elevated transcript expression of PLK1 is associated with worse survival outcomes across all four subtypes of MB. Experiments targeting PLK1 with siRNA knock down- or the kinase inhibitors (i.e. BI-2536 and BI-6727)- can almost completely eliminate MB cell proliferation and colony formation of MB CSCs with concentrations as low as 5–10 nM (Harris et al., 2012; Triscott et al., 2013).

BI-2536 can target MB CSCs as demonstrated through alteration of level of expression of the stem cell gene, SOX2, and the assessment of CD15+ populations (Harris et al., 2012; Markant et al., 2013). Finally, similar to RSK inhibitors, experiments testing normal hNSCs and human astrocytes with therapeutic doses of BI-2536 demonstrate no negative growth effects to normal brain cells (Lee et al., 2012; Triscott et al., 2013). This further supports the idea that new treatments for MB are on the horizon.

21.9 Conclusion

The molecular diversity of brain tumors presents a monumental challenge in treating the disease. Several studies support the idea that normal stem cell pathways are hijacked to initiate the development of brain tumors. The question remains, how to target CSCs as a means to improve patient survival. This could be achieved through multi-targeting approaches as given in the example of DSF. Alternatively, small molecules inhibiting kinases that drive CSCs but not hNSCs may provide a safe mean to treat the cancer while sparing the normal cells. The safety window is especially key in this instance because patients with MB are commonly children who need healthy NSCs for normal brain development.

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22

Induced Pluripotent Stem Cell-Derived Outer-Blood-Retinal Barrier for Disease Modeling and Drug Discovery

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22.1 Introduction

Retinal degenerative diseases (RDDs) are the most common cause of irreversible blindness in the world and, currently, no effective treatments or cures exist. In many cases, RDDs originate in a specialized homeostatic unit located at the back of the eye known as the outer-blood-retinal-barrier (OBRB). The OBRB regulates the microenvironment of the retina and maintains proper fluid and nutrient transport between the photoreceptors and systemic circulation. The functional unit of the OBRB consists of the retinal pigment epithelium (RPE), Bruch's membrane (BM), and fenestrated choriocapillaries. Common RDD pathologies include disruption of the fluid transport capacity of the RPE tissue, neovascularization of the choriocapillaries, and degeneration of the tissues of OBRB. The precise etiologies of many RDDs are still unknown, but the roles of aging and genetics have been extensively studied.

Advanced age is one of the major risk factors for late-onset polygenic RDDs, such as age-related macular degeneration (AMD), and due to the accelerated aging of the US population, the number of individuals diagnosed with AMD in the United States who are 40 years or older is expected to reach almost 3 million by the year 2020 (Friedman et al., 2004). In contrast, monogenic RDDs such as Best Vitelliform Macular Dystrophy (BVMD) begin to manifest during childhood and eventually progresses to significant vision loss by the third to fourth decade of life. Because BVMD is present only in about 1.5 per 100,000 people (Bitner et al., 2012), the rarity of the cases hinders in-depth study of the disease. In addition to the undesirable prognosis and high prevalence, RDDs impose a considerable financial burden on the US economy. Cost associated with blindness

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per year per person is about $13,100 (Köberlein et al., 2013), and AMD alone accounts for a loss of $30 billion annually in the US economy (Brown et al., 2005). There are undeniable health and economic burdens associated with RDDs, and numerous clinical trials are underway to treat patients suffering from these diseases. The three main approaches to treating RDDs are pharmaceutical intervention, gene therapy, and cell transplantation therapy. This chapter will focus on the first approach, specifically on the use of an induced pluripotent stem cell (iPSC)-based outer-blood-retinal-barrier (OBRB) model for ocular drug discovery.

In the US, drug discovery and development is a lengthy and costly process. On average, a pharmaceutical company must spend anywhere from $802 million to $2.56 billion over the course of 13.5 years to get a single drug approved into market (DiMasi et al., 2003, 2016; Paul et al., 2010). The latest study published by DiMasi in 2016 reports that the success rate for a drug getting approved into market is about 11.8% (DiMasi et al., 2016), and this number has been continually decreasing over time (DiMasi et al., 2010). Major causes of a failed clinical trial are the efficacy and safety of drugs (Arrowsmith, 2011a, 2011b), and this suggests a need for improvements in the investigational steps that come prior to clinical trials.

The current paradigm of drug discovery and development includes target and hit identification, lead generation and optimization, preclinical study, and clinical trial (Bleicher et al., 2003). In this process, once an optimized lead compound is identified, it is assayed for efficacy and toxicity on cells (primary or cell lines) or on an animal model. Cell lines are an efficient tool in early stages of drug discovery due to abundant availability, but genetic and epigenetic changes introduced during the induction of immortality and those accumulated during prolonged culture may alter key cellular phenotypes and ultimately the entire model response. Accordingly, it has been shown that a transformed RPE cell lines, ARPE-19 and hTERT, exhibit differential profiling of proteins associated with cytoskeletal remodeling and cell survival compared to that of early passage primary RPE cells (Alge et al., 2006). On the other hand, primary cells obtained directly from a diseased patient may provide a more phenotypically accurate and relevant study model (Unger et al., 2002), but the limited source and regenerative capacity of various cell types, such as RPE, hampers extensive studies on those cell types. Animal models allow for a more comprehensive study on the effects of compounds on the whole body, but physiological responses obtained from animal models aren't necessarily analogous or translatable to the human system (Rice, 2012). Therefore, in order to facilitate a more efficient and effective drug discovery investigation, the disease model must possess human genetics as well as an accurate, structural, and molecular microenvironment of the specific cell type found *in vivo*.

Human pluripotent stem cells (hPSCs) offer a potential solution to generating a more abundant and genetically relevant cell source for human disease modeling and drug discovery. Particularly, the ability to induce cellular pluripotency from a patient's own somatic cells has opened the door to developing a more personalized and accurate disease model. In parallel to the rise of induced pluripotent stem cells (iPSCs), recent advancements in organoid-culture technology and microfluidic chip fabrication technology have allowed for the development of three-dimensional culturing platforms that more closely mimic the macro- and microenvironment found in native tissues or organs. In due time, the integration and synergy of these innovations will aid in reducing the cost and time associated with drug discovery and development. In this chapter, we will discuss the current landscape of iPSC-based OBRB disease models, the engineering of a more relevant culture environment, and their overall implications for drug discovery.

22.2 The Outer Blood-Retinal Barrier

The retina is a neurosensory tissue system responsible for converting visible light into electrochemical signals interpreted by the brain as images. In order to maintain proper neural retinal function, the blood-retinal barrier (BRB) serves to continuously regulate byproducts of the visual cycle and deliver nutrients to satisfy high metabolic

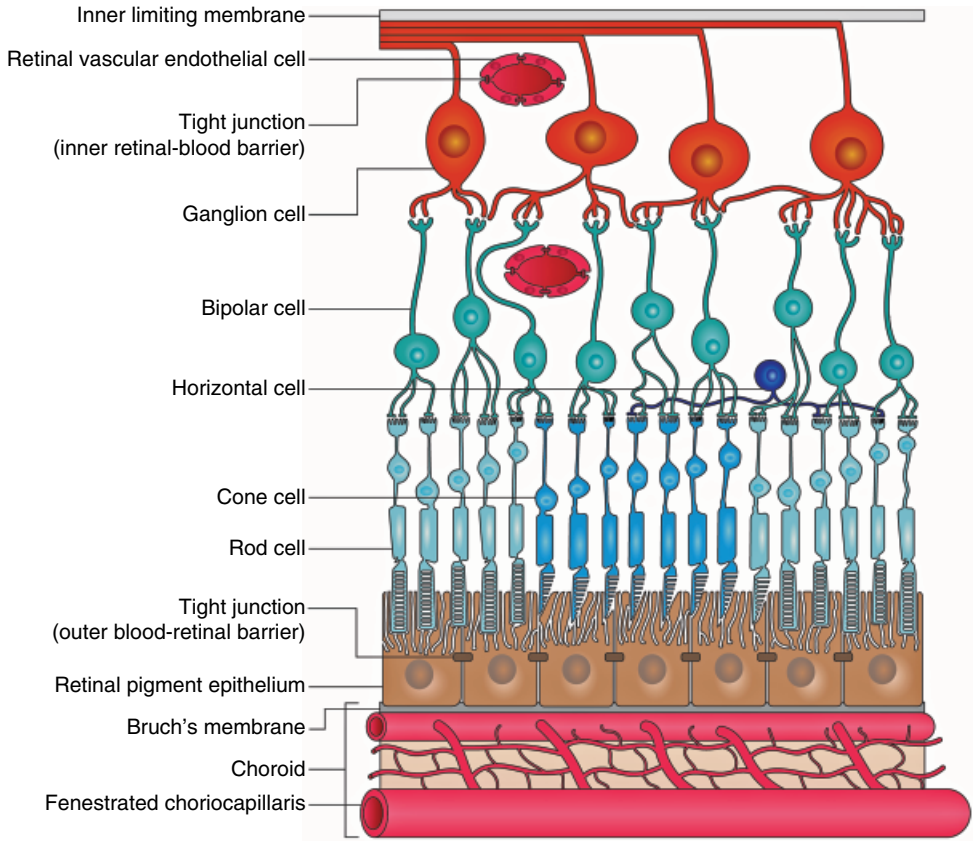

Figure 22.1 ***(Plate 23)*** *Diagram of a healthy human retina and outer blood-retinal barrier. (See insert for color representation of the figure.)*

demand of the retina. Tight junctions of the BRB allow for a very selective control of solute and fluid permeability, and this barrier-like property of BRB is extended to both the inner and outer side of the retina.

The inner BRB (IBRB) is composed of the tight junctions formed between the endothelial cells of the retinal blood vessels that primarily exist in the inner plexiform and ganglionic layers of the retina (Figure 22.1/Plate 23). Regulatory signals from astrocytes, pericytes, and Muller cells that surround the retinal vascular endothelial cells influence the permeability of the retinal blood vessels in selectively transporting fluid and nutrients between the inner retina and the vascular lumen. Dysfunction of this retinal microvasculature is associated with diabetic retinopathy, which affects a third of the patients suffering from diabetes mellitus (Kempen et al., 2004), and retinopathy of prematurity (Bharadwaj et al., 2013).

The outer BRB (OBRB) is located on the side opposite of the IBRB with respect to the neural retina, and the key component of OBRB is the retinal pigment epithelium (RPE) situated between the neural retina and Bruch's membrane. The RPE is composed of a monolayer of RPE cells joined together by tight junctions and it regulates the transport of ions, nutrients, and metabolites between the retina and the fenestrated choriocapillaries located on the opposite side of the Bruch's membrane. In addition to its barrier function, the RPE is responsible for maintaining the health and function of the retina. When a photon enters the eye and is absorbed by a photoreceptor, the visual chromophore 11-*cis*-retinal is photoisomerized to all-*trans*-retinal. This converted

product is then transported to the RPE, re-isomerized back to 11-*cis*-retinal, and transported back to the photoreceptor to prepare the photoreceptor for another round of neural excitation. Furthermore, the photo-oxidized photoreceptor outer segment (POS) of photoreceptors are continually phagocytized by the RPE, which recycles and returns essential components to the photoreceptors for rebuilding their outer segments. The RPE is also able to help maintain the functional and structural integrity of the photoreceptors and the choriocapillaries by secreting essential growth factors, such as pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF), in a polarized fashion. Lastly, the RPE is implicated in establishing and maintaining the immune privilege of the eye through the secretion of immunosuppressive factors (Shi et al., 2008).

The RPE, Bruch's membrane, and the choriocapillaries together form a functional unit responsible for maintaining the structural and functional integrity of the outer retina. Many key functions of RPE depend on its connection to the systemic circulation through the pentalaminar Bruch's membrane, which plays a crucial role in cell proliferation, differentiation, and cellular communication (Campochiaro et al., 1986; Guymer et al., 1999; Olson, 1979). The highly permeable capillary network of the choriocapillaries is crucial in delivery of nutrients and oxygen to the retina and for removal of waste products generated by photoreceptors and the RPE (Lutty et al., 1999). Overall, a complex synergy within this functional unit is crucial in maintaining the homeostasis of the retinal environment, and consequently, a failure in any part can lead to retinal degeneration. Therefore, a comprehensive study of OBRB requires the attention of not only the RPE, but also its surrounding components.

22.3 iPSC-Based Model of the Outer-Blood-Retinal-Barrier

22.3.1 Stem Cell Technology Overview

In 2006, Shinya Yamanaka successfully generated pluripotent stem cells from embryonic and adult mouse fibroblast (Takahashi and Yamanaka, 2006). The reprogramming of somatic cells into pluripotency was made possible by overexpressing four key transcriptional factors: Oct4, Sox2, Klf4, and c-Myc, now known as the Yamanaka factors. Subsequently, Yamanaka and others extended this discovery by generating the first human induced pluripotent stem cells (hiPSC) from human fibroblasts (Takahashi and Yamanaka, 2006; Yu et al., 2007). Since then, alternative protocols were developed to improve the efficiency and safety of the reprogramming process (Hochedlinger and Jaenisch, 2015). The method of introducing the transcription factors initially involved genome-integrating viruses, which could potentially introduce insertional mutations and alter the overall differentiation potentials of the target cells. A known proto-oncogene, c-MYC, was phased out of the reprogramming process in order to eliminate the possibility of tumorigenesis from its reactivation (Yu et al., 2007). Integration-free method of iPSC generation has also been widely investigated using Sendai virus, modified mRNA, micro-RNA, and small-molecular compounds (Hochedlinger and Jaenisch, 2015).

Specific cell types differentiated from a patient-derived iPSCs offer advantages over traditional primary cell and embryonic stem cell (ESC) based disease models and therapies. One key disadvantage of primary cell models derived from donor tissue is the inevitability of batch-to-batch variation inherent in acquired samples. For iPSC-derived models, a robust differentiation protocol may minimize any quality variation between derived tissues. Additionally, primary cells lack the ability to continually self-renew. In contrast, iPSCs provide unlimited source of patient-derived, disease-specific cells that can be differentiated into desired cell types. In terms of obtainability, primary cells from tissues in the back of the eye or the central nervous system are difficult to acquire and maintain in culture, which may hinder prolonged, extensive studies. Furthermore, primary cells are often harvested after disease onset, but iPSC-based models allow for the observation of disease development from earlier induction stages.

The similarities and differences between hiPSCs and hESCs have been a subject of intense study since the development of hiPSCs. An early study by Chin et al. revealed that ESCs and iPSCs have different gene expression profiles, suggesting different epigenetic state of the two cell types (Chin et al., 2009). In contrast, a later study by Bock et al. compared genome-wide reference maps of DNA methylation and gene expression of 20 hESC lines and 12 hiPSC lines and did not reveal a distinct pattern that distinguished the two different kinds of hPSC lines (Bock et al., 2011). The use of hESCs for research has been a topic of a heated ethics and policy debate, and the risk of immune rejection imposes considerable limits on hESC research over that of hiPSC. By using iPSC-based disease models obtained directly from patients, clinicians, and scientists are able to better study and understand the relationship between the clinical disease phenotype and its underlying cellular mechanisms.

22.3.2 Optimization of RPE Differentiation

Perhaps the most important aspect of an investigation utilizing an iPSC-derived OBRB disease model is a robust protocol for consistently and sufficiently generating functional RPE cells. Initial effort to derive RPE from PSCs employed mouse and primate ESCs cocultured with PA6 mouse skull stromal cell line (Hirano et al., 2003; Kawasaki et al., 2002), and the protocol continued to improve with the use of human PSCs. Strategies used over the years to differentiate hPSCs into RPE are classified into two broad categories: spontaneous and directed differentiation. Early studies indicated that in the absence of external inductive cues, ESCs commit to the neural pathway for differentiation (Muñoz-Sanjuán and Brivanlou, 2002). RPE is one of the derivatives of neuroectoderm, a progenitor of neuronal lineage, and accordingly, Klimanskaya et al. were able to manually isolate and culture spontaneously differentiated RPE cells from an overgrown hESC culture deprived of basic Fibroblast Growth Factor (bFGF) (Klimanskaya, 2006). Although reliable and simple in design, the spontaneous differentiation method is not very efficient and requires at least a month before initial pigmentation even appears in culture. Additional 1–2 months are required for the pigmented foci to become large enough to be manually isolated and expanded. Furthermore, the small RPE culture obtained initially may require a high level of expansion to achieve desired amount of cells, and this process compromises the purity of the final product by increasing the likelihood of selecting for non-RPE cell types and by inducing epithelial to mesenchymal transition in RPE resulting in their loss of epithelial phenotype (Croze et al., 2014; Singh et al., 2013a). The spontaneous differentiation method has been shown to be applicable for iPSCs as well, and the derived RPE showed RPE-specific gene expression profile and POS phagocytosis ability (Buchholz et al., 2009; Carr et al., 2009a; Hu et al., 2010).

The other method of generating RPE cells from PSCs is closely associated to the *in vivo* embryonic development of the eye. In directed differentiation of RPE, specific exogenous agents are added at different time points to sequentially induce the formation of neuroectoderm, optic cup progenitors, immature RPE-like cells, and mature RPE (Ferrer et al., 2014; Hirami et al., 2009; Meyer et al., 2009; Osakada et al., 2008, 2009). Studies demonstrate that this retinal progenitor induction is attributable to a combination of WNT, NODAL, and TGF pathways (Chambers et al., 2009; Meyer et al., 2009; Osakada et al., 2009), and subsequently, RPE differentiation can be preferentially induced by activating the TGF-b or WNT pathways (Idelson et al., 2009; Leach et al., 2015). The overall efficiency of RPE generation is much greater using the directed differentiation method compared to the spontaneous differentiation method (Klimanskaya et al., 2004; Osakada et al., 2008, 2009), but the lack of general consensus on which pathways to target during differentiation may hinder the development of an effective, standard protocol. Later studies investigated ways to enhance the clinical applicability of differentiated RPE using xeno-free or small-molecule-based protocols (Maruotti et al., 2015; Osakada et al., 2009). Table 22.1 identifies a list of publications that have investigated the differentiation of RPE using iPSCs.

Table 22.1 *Derivation of retinal pigment epithelium from human induced pluripotent stem cells*

References	Duration	Differentiation enhancement strategy	Functional Tests	Application
Spontaneous Differentiation				
(Buchholz et al., 2009)	>90 days	bFGF removal and mechanical isolated for enrichment	Bovine POS phagocytosis	N/A
(Carr et al., 2009b)	>120 days	bFGF removal and mechanical isolated for enrichment	Porcine POS phagocytosis	Transplantation into retinal dystrophic rat
(Hu et al., 2010)	>60 days	bFGF removal and mechanical isolated for enrichment	N/A	N/A
Directed Differentiation				
(Buchholz et al., 2013)	2–8 weeks	Activin A, SU5402, IGF1, Noggin, Dkk1, bFGF, VIP, NIC added at appropriate times	POS phagocytosis	Rapid RPE generation
(Ferrer et al., 2014)	>7 weeks	Dual SMAD inhibition, Activin A, and nicotinamide	N/A	High throughput screening
(Hirami et al., 2009)	>40 days	Dkk-1 and Lefty-A	N/A	RPE generation from iPSC
(Maruotti et al., 2015)	>60 days	Chetomin and nicotinamide	POS phagocytosis and polarized VEGF/PEDF secretion	Small molecule induction
(Osakada et al., 2009)	>90 days	CKI-7 and SB-431542	POS phagocytosis	Small molecule induction

In order to ensure the consistency and validity of OBRB-related investigations, the proper authentication of RPE derived from an iPSC must precede any effort to utilize the cells for disease modeling, drug screening, or transplantation. The semi-quantification of POS phagocytosis is the most well-known functional assay used for RPE PSC-derived authentication. Immunohistochemistry for ZO-1 and RPE65, observation of characteristic hexagonal morphology, and proper transepithelial resistance are widely used to assess the maturity of RPE tissue. In addition, Ferrer et al. investigated two new physiologically relevant measures of RPE authenticity: response of cytoplasmic calcium concentration to external purinergic stimuli and changes in membrane potential in response to changes in extracellular potassium concentration, both of which occur in the sub-retinal space in response to a light stimulus (Ferrer et al., 2014). The utilization of these functional and molecular assays to develop a standardized benchmark for RPE maturity is critical to furthering inter-laboratory result compatibility and reproducibility, and to develop assays that can be used to test drug toxicity on cells.

22.3.3 Development of the Homeostatic Unit of the OBRB

The development of the complete OBRB homeostatic unit model, which consists of the RPE, Bruch's membrane, and choriocapillaris, is crucial to studying RDDs related to OBRB. Choriocapillaris is the fenestrated vascular network that supports the outer retina through nutrient and waste exchange, and it is composed of choroidal endothelial cells (CECs) and choroidal pericytes. The advent of stem cell technology has allowed for the generation of vascular cells using both hESCs and hiPSCs, but initial methods were inefficient and required coculture with OP9 feeder cells (Choi et al., 2009; Sone et al., 2007; Taura et al., 2009). Later monolayer-directed differentiation methods have allowed for a much more efficient and rapid generation of

endothelial cells by using selective GSK3β inhibition induced WNT signaling activation for mesoderm induction with subsequent vascular specification using TGF-β pathway inhibition and VEGF-A addition (Orlova et al., 2014; Patsch et al., 2015). Despite extensive development in vascular differentiation, little progress has been made thus far on methods specific to generating CECs. Recently, Songstad et al. have demonstrated the feasibility of CEC-specific differentiation, but the protocol lacks clinical applicability due to its need for coculture with monkey CEC cell line or mouse primary CECs. While choriocapillaris is distinguished from other vascular tissues by the expression of choroid-specific markers, such as carbonic anhydrase 4 (CA4) and plasmalemma vesicle associated protein (PLVAP), further study is required to establish whether a CEC-specific culture is necessary for studying RDD pathologies in OBRB-based disease models.

The Bruch's membrane, named after the German anatomist Karl Wilhelm Ludwig Bruch, is a dynamic pentalaminar barrier situated between RPE and the choriocapillaris, and it is a crucial component of the RDD model due to its unique location, function, and structure (Booij et al., 2010). BM is composed of (from most anterior to posterior) the basement membrane of RPE, the inner collagenous layer, a porous elastic layer, the outer collagenous layer, and the basement membrane of the endothelium of the choriocapillaries (Hogan, 1961). The key functions of BM include, but not limited to, the regulation of fluid and material exchange between RPE and the choriocapillaris, providing support for RPE adhesion (Del Priore et al., 2002) and perhaps differentiation (Gong et al., 2008), restricting retinal and choroidal cellular transmigration, and wound healing (Tezel et al., 2004). Since BM itself doesn't contain any cells, passive diffusion is the primary method of transport through the membrane (Grindle and Marshall, 1978), and the rate of transport is directly influenced by its structural and molecular composition, which has been shown to be associated with age, genetics, and disease state (Bhutto et al., 2008; Curcio et al., 2001; van Soest et al., 1997). Since the native BM is difficult to obtain and the protocol for its *in vitro* synthesis is still unknown, potential substitute materials such as the decelluarized amniotic membrane and fibroin, a silk protein, have been investigated (Hamilton and Leach, 2011; Shadforth et al., 2012). Given the positive findings from these initial studies, the recapitulation and incorporation of BM to OBRB model is crucial and inevitable to developing a more accurate and relevant RDD model.

22.4 iPSC Based OBRB Disease Models

The purpose of a biological model is to recapitulate the state and function of a given native system. In the past, primary cells, immortalized cells lines, and animal models had been the gold standard of modeling a particular cell, tissue, or organ in the human body. However, the overall lack of consistency, abundance, and geno/phenotype relevance of these models have often failed to accurately relate their findings to the native human system. Understandably, these model systems by definition are an analogy – the quality of which will always approach, but never quite arrive at, the asymptote that is the human itself. Nevertheless, recent discoveries relating to the induction of human somatic cells to pluripotent state have created the possibility of generating an infinite supply of patient- and disease-specific cells to model any part of the body. Additionally, the advancements in the field of biologically inspired engineering have allowed for the development of model systems that more closely mimic the three-dimensional structure of our native tissues or organs. Developing a more accurate model system gives rise to a more real disease state simulation. Ultimately, the goal of a disease model should be to: (1) determine the genetic and cellular phenotype associated with the disease; (2) elucidate molecular mechanisms and pathways related to the disease pathogenesis; (3) develop relevant targeted interventions to cure or to alleviate the abnormalities in the model; and (4) translate such therapies to humans. In this section, we describe studies that utilize patient-specific iPSC-RPE to achieve these goals with respect of RDDs.

22.4.1 Two-Dimensional iPSC-RPE Disease Models

Two-dimensional disease models offer a simplified view of disease state by oftentimes limiting the scope of the model to a single functional unit of tissue. Although somewhat limited in its ability to observe macro-level, systemic responses, isolated two-dimensional models allow for efficient investigations in cause-and-effect relationships to elucidate key molecular mechanisms related to disease state. Table 22.2 identifies various RDD models utilizing two-dimensional iPSC-RPE culture.

Table 22.2 *Ocular disease models utilizing iPSC-derived RPE*

Disease	Genetic/phenotypic abnormalities observed	Genetic correction	Drug test	References
Gyrate atrophy	Low ornithine-δ-aminotransferase (OAT) activity	*OAT* transfer via BAC-medicated homologous recombination	Vitamin B_6	(Meyer et al., 2011)
Leber congenital amaurosis	Mutation at *GUCY2D*, upregulation of NNAT, and downregulation of *GSTT1, TRIM61, ZNF558*	No	No	(Lustremant et al., 2013)
Retinopathy	Mutation of *HADHA* and hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency	No	No	(Polinati et al., 2015)
Choroideremia	Mutations in *CHM* gene encoding *REP1*	AAV2/5-mediated *CHM* transfer	No	(Cereso et al., 2014)
Retinitis pigmentosa	Mutation of gene *R2* and defects in IFT20 localization, Golgi cohesion, and Gβ1 trafficking.	No	G418 and PTC124 (TRIDs)	(Schwarz et al., 2015)
	Mutation of *MFRP* and disrupted RPE pigmentation and transepithelial resistance	AAV8 (Y733)-mediated *MFRP* transfer	No	(Li et al., 2014)
	Ser331Cysfs*5 mutation in *MERTK*	No	No	(Lukovic et al., 2015)
Age-related macular degeneration	Upregulation of *KEAP1, NQO1, HMOX1, GCLC*, and *GCLM*	Lentivirus-mediated has-miR-144 overexpression	Ai-1	(Garcia et al., 2015)
	Lower expression of antioxidant genes *HO-1, SOD2*, and *GPX1* and higher accumulation of ROS compared to control	No	Curcumin	(Chang et al., 2014)
	ARMS2/HTRA1 risk alleles decreased SOD2 defense	No	No	(Yang et al., 2014)
Best vitelliform macular degeneration	Mutation at *BEST1*, disrupted fluid flux of BRB, and increase accrual of autofluorescent material	No	Valproic acid	(Singh et al., 2013b, 2015)
Autosomal recessive betrophinopathy	R141H and I366fsX18 mutation at *BEST1*	No	No	(Johnson et al., 2015)
Usher syndrome	Mutation at *USH2A* and upregulation of *GRP78* and *GRP94*	No	No	(Tucker et al., 2013)
Leber's hereditary optic neuropathy	14484T to C point mutation of *ND6*	No	No	(Zahabi et al., 2011)

22.4.1.1 Pigment Retinopathy

Homozygous mutation in the trifunctional protein α-subunit gene *HADHA* (hydroxyacyl-CoA dehydrogenase) is the most common cause of long-chain 3-hydroxyl-CoA dehydrogenase (LCHAD) deficiency, which typically manifests into pigment retinopathy (Tyni and Pihko, 1999). If not detected early, retinopathy may lead to chorioretinal atrophy, occlusion of choroidal vessels, development of posterior staphylomas and central scotomas, developmental cataract, myopia, and degeneration of central vision (Tyni et al., 1998). Polinati et al. were the first to utilize iPSC-RPE to investigate LCHAD pigment retinopathy disease model *in vitro* (Polinati et al., 2015). Although the exact pathogenesis of pigment retinopathy remains unclear, Polinati et al. succeeded in recapitulating the cellular disease phenotype. Compared to RPE derived from a control patient, the RPE-based retinopathy model revealed disrupted RPE cell morphology and disorganized cell-cell tight junctions, which could lead to abnormal OBRB functions. Additionally, the diseased cells showed relatively fewer melanosomes and greater accumulation of long-chain triglycerides, indicating reduced oxidant scavenging potential and lipid toxicity of RPE (Polinati et al., 2015).

22.4.1.2 **Gyrate** *Atrophy*

Gyrate atrophy (GA) is a rare autosomal recessive disease characterized by RPE and photoreceptor loss. One of the characteristics of GA is a defect in the gene encoding ornithine-δ-aminotransferase (OAT), which is responsible for converting L-ornithine into neurotransmitter GABA (Stránská et al., 2008; Wang et al., 2000). A study done using a mouse model related ornithine accumulation as one of the factors in the pathophysiology of GA (Wang et al., 2000). In 2011, Meyer et al. established one of the first patient-specific RDD models using iPSCs to not only recapitulate key GA cellular phenotypes similarly observed in the mouse model, but also use the model to identify possible pharmaceutical or gene therapy solutions (Meyer et al., 2011). The study generated iPSCs from GA patients with OAT mutation and differentiated them into RPE cells which displayed very low OAT activity. Subsequently, they were able to rescue the *OAT* activity by using a high dose of vitamin B_6 or by repairing, through bacterial artificial chromosome (BAC)-mediated homologous recombination, the *OAT* gene of hiPSCs before RPE differentiation. Although the complete pathogenesis of GA remains unclear, this study demonstrated the feasibility iPSC-based studies for GA as well for other RDDs.

22.4.1.3 Choroideremia

Choroideremia is an X-linked recessive inherited disorder that results in the degeneration of the choriocapillaries and RPE, eventually leading to blindness. A single gene, *CHM*, which encodes Rab escort protein-1 (REP1), a chaperone protein that phenylates Rab guanosine triphosphatases (GTPases) and delivers them to the membrane, has been implicated for the cause of choroideremia. The study of this disease using animal models proved to be challenging, as the REP1 mutation or knockout is lethal for both mouse and zebrafish (van den Hurk et al., 1997; Starr et al., 2004). In 2014, Cereso et al. developed a choroideremia disease model using patient-specific iPSCs (Cereso et al., 2014). The study utilized functionally validated, iPSC-differentiated RPE to detect underlying biochemical defects and study gene transfer therapy. Assays revealed that RPE cells derived from choroideremia patients showed significantly higher ratio of cytosolic to membrane-bound Rab27A content compared with that in wild-type RPE. Subsequent transduction of *CHM* using AAV2/5 vector normalized REP1 expression and membrane-bound Rab27A level of diseased RPE cells. The study provided one of the earliest proof of concept for AAV-mediated gene transfer therapy on human cellular model of the diseased retina.

22.4.1.4 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries, and despite extensive investigations its pathogenesis remains unclear thus far. While the causes of many other RDDs can be isolated to mutations in a handful of genes, over 50 different loci with varying degree of disease causing risk have been identified in patients with AMD (Fritsche et al., 2014). Therefore, conducting extensive studies through abundant and genetically relevant sources is crucial to identifying possible pathological mechanisms of AMD. Yang et al. demonstrated the feasibility of modeling polygenic disease like AMD by identifying a possible downstream target of ARMS2/HTRA1 risk alleles (Yang et al., 2014). The study utilized three patient cell lines specifically genotyped for a combination of AMD-associated risk haplotype (T-in/del-l-A) and observed impaired superoxide dismutase 2 (SOD2) activity, which mediates antioxidative defense, in RPE homozygous for the highest risk haplotype. Although the study falls short in elucidating a specific molecular pathway associated with abnormal ARMS2/HTRA1 activity, it was relatable to previous animal model findings (Kasahara et al., 2005; Sandbach et al., 2001). Chang et al. observed similar SOD2 impairment in AMD patient-derived RPE cells and was able to test the efficacy of curcumin in restoring anti-oxidative defense (Chang et al., 2014). Similarly, Garcia et al. tested the therapeutic potentials of a microRNA and a small molecule modulator of NRF2-KEAP1 pathway in alleviating oxidative stress (Garcia et al., 2015). However, further investigation on the effects of long-term NRF2 pathway activation is needed to establish stronger clinical applicability.

22.4.1.5 Bestrophin-Related Diseases

The gene *BEST1* encodes bestrophin-1 (BEST1), a calcium-activated anion channel primarily localized to the basolateral plasma membrane of RPE cells (Marmorstein et al., 2009). BEST1 serves key roles in regulating intracellular calcium signaling and calcium homeostasis (Marmorstein et al., 2015), and its dysfunction has been closely associated with RDDs such as autosomal recessive bestrophinopathy (ARB) and BVMD. BVMD is an early-onset RDD that causes progressive vision loss due to the dysfunction of RPE, and it is the most prevalent disease caused by *BEST1* mutation. The BVMD model made using the mutant iPSC-RPE displayed relatively higher-level disruption in fluid transport, POS degradation, protein homeostasis, and calcium and oxidative stress regulation (Singh et al., 2013b, 2015). While the cellular phenotype observed using this model were relatable to the clinical phenotype of BVMD, further studies are needed to determine the specific pathways linking *BEST1* mutations to the disease cellular phenotype. Rather than molecularly linking the mutation to a cellular pathway, Singh et al targeted one of the cellular phenotype – defective protein homeostasis (Singh et al., 2015). Authors demonstrated that treatment of patient cells with Valproic acid diminishes proteostasis toxicity in cells and increased POS degradation. Similar to BVMD, ARB is characterized by retinal detachment, subretinal deposits and fluid accumulations, and causes significant vision loss as early 4 years of age (Boon et al., 2009; Marmorstein et al., 2009). Limited knowledge regarding the role of BEST1 in ARB pathogenesis hinders the development of effective therapies. Toward that end, a study conducted by Johnson et al. using iPSC-RPE discovered a novel mutation associated with ARB and contributed to a clearer understanding of the function of BEST1 in relation to its structure (Johnson et al., 2015). The study found that a previously described *BEST1* mutation R141H (Burgess et al., 2008) alone is not enough to cause ARB, and that the coexpression of both R141H and I3666fsX18, a mutation that disrupts the C-terminus of BEST1, results in ARB.

22.4.1.6 Leber Congenital Amurosis

Leber congenital amurosis (LCA) is a rare, autosomal recessive disorder that usually manifests around the age of 6 weeks, and it is the most severe form of inherited retinal blindness (den Hollander et al., 2008). In an effort to find genetic correlations associated with LCA, Lustremant et al. used LCA patient-derived

iPSCs to run genome-wide transcriptome analysis. The results revealed a common polymorphism on the *GUCY2D* gene, associated with the LCA type I, but the causative gene is still unknown (Lustremant et al., 2013). Furthermore, RPE cells differentiated from LCA patient-derived iPSCs exhibited higher expression of *NNAT* gene and reduced expression of *GSTT1, TRIM61*, and *ZNF558* genes, offering a possible mechanistic explanation for GUCY2D mutation induced LCA.

22.4.1.7 Retinitis Pigmentosa

Retinitis pigmentosa (RP) is the most common type of early-onset hereditary retinal degeneration, globally affecting about 1 in 4000 individuals (Hartong et al., 2006). More than 60 genes inherited as autosomal dominant, autosomal recessive, or X-linked have been implicated for association with RP (Sahel et al., 2010). Although genetically heterogeneous, RP patients display common clinical phenotypes, such as punctuate pigmentation, thin retinal vessels, dysfunction of the photoreceptors and/or RPE, and progressive photoreceptor degeneration (Berger et al., 2010). Schwarz et al. focused the investigation around a RP model harboring a R120X mutation in the *RP2* gene and looked for a potential therapy (Schwarz et al., 2015). RP2 protein was completely absent in both iPSCs and iPSC-RPE cells derived from the RP2 patient, and the model created using iPSC-RPE showed phenotypic abnormalities in intraflagellar transport (IFT20) particle localization, Golgi cohesion, and Gβ1 trafficking. Overexpression of RP2-GFP rescued the defects, indicating that observed phenotypes were a consequence of loss of RP2 protein. Since the mutation was a nonsense mutation, treatment with translational read-through inducing drug (TRID), G418, caused a 20% increase in intracellular RP2 protein level of the R120X iPSC-RPE cells, and this level of restoration was enough to rescue the phenotypic defects. Li et al. studied RP in related to the defects in Membrane Frizzled-related Protein (MFRP) (Li et al., 2014). The genetic rescue of *MFRP* gene using AAV8 vector normalized RPE pigmentation and transepithelial resistance, demonstrating the potential of AAV-mediated gene therapy for treating MFRP-related RP. The study also translated the finding to *MFRP* mutant mice and observed long-term improvement in their visual function. Lukovic et al. generated a model for RP caused by the mutation of mer tyrosine kinase receptor (MERTK) (Lukovic et al., 2015). RPE cells differentiated from patients with Ser331Cysfs*5 mutation in *MERTK* gene showed defective phagocytosis, consistent with the phenotype observed in animal model. Although the study did not pursue potential therapies targeted towards rescuing the *MERTK* gene, the model provides a robust cellular model for future studies regarding therapies for RP.

22.4.2 Development of Three-Dimensional Models

While two-dimensional models provide a simplistic approach to efficiently carrying out studies on a specific type of cells, the design inherently limits the accommodation of more than one type of cells and is often unable to mimic key structural definitions surrounding the cells. As the pathogeneses of RDDs are closely related to the OBRB as a whole, the development of a more complex and relevant cellular environment is crucial to accurately modeling RDDs. For example, polygenic diseases such as AMD will require not only an abundant supply of AMD patient-specific iPSCs to elucidate the associated abnormalities, but also a more comprehensively appropriate model that can simultaneously recapitulate the effects of the environment within and surrounding the outer blood-retinal barrier. Specifically, an ideal OBRB model system must be able to integrate micro-environmental factors such as the pressure exerted from the eye's interior, the heterogeneity of tissue composition between the macula and the periphery, the structure and composition of the extracellular matrix surrounding the OBRB, and the specific autocrine, paracrine, and endocrine signaling molecules that transverse the tissue system. Toward these goals, both organoids and microfluidic culture systems hope to bridge the gap between the two-dimensional culture and the native environment of the

human body. This section will provide a brief overview of the evolution of three-dimensional tissue culture systems and their potential applications for OBRB models.

22.4.2.1 Autonomous Self-Assembly

One approach to developing a three-dimensional (3D) tissue or organ model involves *in vitro* induction of the embryonic development process. Organoids are self-assembled and self-organized structures derived from PSCs or organ progenitors through developmentally guided cues, and resulting structures exhibit both organ-specific cell types and structurally relevant definitions (Lancaster and Knoblich, 2014). The brain, intestine, and the eye were the earliest organoid models developed from hPSCs, and both structures exhibited relevant spatial organization and cellular composition (Eiraku et al., 2008, 2011; Spence et al., 2011; Suga et al., 2011). Specifically, the *in vitro* development of the optic cup using hESCs by Eiraku et al. laid out the groundwork for subsequent studies regarding 3D ocular models (Eiraku et al., 2011). Later studies have successfully replicated and improved upon the original findings and translated the method to be compatible with iPSCs (Nakano et al., 2012; Phillips et al., 2012; Reichman et al., 2014; Zhong et al., 2014). Overall, the investigations on developing 3D ocular models have led to greater understanding of the ocular organogenesis and have contributed to the development of more efficient and effective developmentally guided differentiation protocols for generating cells of the retina. However, the ability to generate only organ-specific cell types in organoid models is a double-edged sword. While many RDDs have been associated with specific genetic mutations that contribute to phenotypic defects in cells derived from retinal progenitor cells, the pathogeneses of RDDs often also involve the surrounding tissues and structures (e.g., choroidal neovascularization). Since the generation of organoids involve the promotion of a specific germ layer related to the organ of interest, organoid-based disease models lack the ability to study disease-specific contributions from related, supporting tissues derived from other germ layers (e.g., RPE of ectoderm and endothelial cells of endoderm). Moreover, many experimental parameters of organoid culture systems are difficult to consistently replicate and quantify as organoids are variable in size and shape. The inability to accurately quantify key organ and tissue characteristics, such as absorption and secretion of specific molecules, from organoid systems may also hinder accurate disease modeling.

22.4.2.2 Engineering Intervention

The synergy of engineering and biology have shown to remedy some of the issues associated with self-assembled 3D organ models. Specifically, the microfluidic culture platform has been the subject of recent investigations due to its success in recapitulating tissue or organ-level structure and functions within a small, polymer-based chip (Huh et al., 2010, 2011). Perhaps the greatest advantage unique to chip-based models is the ability to closely observe and manipulate the microenvironment of the cells at unprecedented resolutions. The chambers that house the cells inside the chip can be strategically arranged and interfaced with each other to mimic the native tissue-tissue interfaces unique to each organ, which allows the cells to proliferate and organize themselves in a more relevant manner (Figure 22.2, later, shows an example of such device for OBRB). The flexibility inherent in the design process of the chips allows for the integration of various microsensors that can be used to analyze key parameters such as tissue barrier integrity and fluid dynamics of the microenvironment. In addition, the ability to continuously perfuse the chambers with fresh media and collect their outputs not only allows for certain cell types, such as endothelial cells, in culture to experience shear stress much like that of their native environments, but also allows researchers to serially collect specific amount of media to quantify and track the amount of secreted proteins or molecules throughout disease progression. A landmark study conducted by Huh et al. functionally and structurally recapitulated the alveolar-capillary interface to model the human lung on a microfluidic chip (Huh et al., 2010), and subsequently used the same lung-on-a-chip

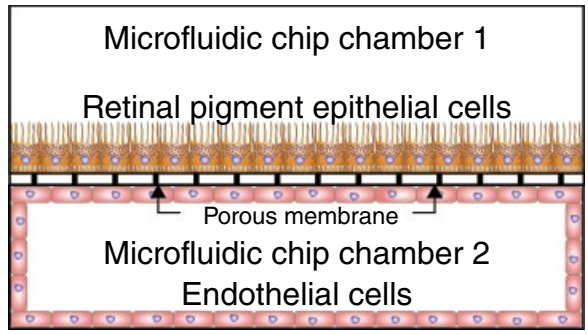

Figure 22.2 ***(Plate 24)*** *Example of an OBRB model in a microfluidic chip with RPE cells in chamber 1 separated by a porous membrane from chamber 2 with endothelial cells. (See insert for color representation of the figure.)*

to model drug toxicity-induced pulmonary edema, demonstrating the potential of organ-on-a-chip for drug discovery and disease modeling (Huh et al., 2012). The advantages and flexibility associated with microfluidic platform have led to the creation of numerous other tissue and organ models (Bhatia and Ingber, 2014), and given the current progress, the development of a chip-based model specific to OBRB for observing RDDs is reachable in the foreseeable future.

22.5 Applications of iPSC-Based Ocular Disease Models for Drug Discovery

The high cost, slow development, and low success rate of drugs that show pre-clinical promise is caused by the inability of current *in vitro* (immortalized cell lines) or *in vivo* (animal) models to recapitulate human disease physiology effectively. As discussed above, *in vitro* cultures are frequently immortalized cells of a specific tissue. Although the ability to replicate indefinitely is good for comparability of research, it is often unknown if any molecular pathways have been disrupted as a cost for immortality. This hinders the process of elucidating the disease phenotype since the resulting intracellular environment is not a true recapitulation of a diseased cell. Additionally, systemic effects of drugs/therapies are unknown using these models. As a means to assess systemic effects researches currently use animal models. However, research has shown that animal models frequently respond differently to drugs or gene therapy than their human counterparts (Nguyen et al., 2015b; Pound et al., 2004; Rice, 2012; Seok et al., 2013). Although they are valuable in that researchers are able to see the response of a therapy at the whole organism level, the inherent differences in the genetic and epigenetic background between humans and non-humans confers a large caveat to any animal model based study. To overcome these limitations scientists are developing a variety of testing platforms that can assess primary, patient specific cells in a cost effective and efficient manner so as to make drug testing commercially feasible. Furthermore, iPSC-based and tissue engineering based approaches open up the possibilities of providing drug testing that is patient specific and provides preclinical data that is potentially more relevant for the clinical use of the drug.

22.5.1 High-Throughput Drug Screening

The ultimate goal of high-throughput (HTP) screening using iPSCs is to be able to test drugs on a variety of disease-specific cells/tissues differentiated from a bank of iPSCs that represent a large patient population. This will provide data on a system-level response of a potential therapy on multiple individuals affected with a given disease. This goal has been suggested for drug discovery and for personalized medicine across many

fields of biology (Charbord et al., 2013; Inoue and Yamanaka, 2011; Maury et al., 2012). However, there have been few studies relevant for ocular diseases that have used iPSCs for drug discovery. Of the studies that have been published no direct clinical trials have been spurred but promising leads have been discovered. Examples include the ability to restore ornithine amino transferase activity in iPSC-derived RPE affected with gyrate atrophy using vitamin B6 (Meyer et al., 2009); increasing Rhodopsin expression in photoreceptors derived from retinitis pigmentosa patients with α-tocopherol treatment (Jin et al., 2011); and rescuing protein homeostasis in BEST disease patient RPE cells using Valproic acid (Singh et al., 2015). These studies provide the proof of principle that iPSC technology can be used for drug testing across related, yet genetically distinct, ocular diseases. More encouragingly, a HTP methodology to differentiate, expand and authenticate iPSC-RPE in parallel to their use in physiological assays has recently been developed (Ferrer et al., 2014). In this assay the RPE cells were derived from iPSCs and grown in 96- and 384-well plates. The researchers used a semi-automated multiplex gene expression assay to demonstrate differential expression of eight distinct genes in iPSCs and iPSC-derived RPE at two differentiation stages, and compared this data to primary human RPE. The multiplex assay was able to clearly identify two different developmental stages of iPSC-derived RPE compared to primary human RPE cells. This study showed that such multiplex gene expression assays could be used to perform drug screens targeted at cell maturity and for targeting the expression levels of genes directly related to disease phenotype. With the proof-of-principle drug studies and this HTP assay, the foundation to create novel HTP drug screening technologies for the OBRB has been formed. The combination of drug screening feasibility and validated HTP assay also creates a framework for how future studies into other ocular tissues should be developed.

It is worth noting that potential hurdles for future ocular related HTP screening tests using iPSCs involve several common HTP challenges. By necessity, good HTP screening tests are inexpensive, fast, and simple. However, tissues are structurally and behaviorally complex and thus finding fast and simple assays that can recapitulate or assess this complexity is challenging. Most HTP assays to date are unable to capture the three-dimensional environment present in a native tissue or organ, which could lead to inaccuracies in the model in terms of drug responses or expressions of disease phenotypes (Lehr, 2005). Additionally, because the tests are fast, long time scale events that are critical to tissue performance cannot be easily assessed. Finally, simple systems are usually unable to recapitulate the complexity of the multi-tissue and organ microenvironments inherent in native tissue organization. Therefore, one envisions that initial large-scale HTP screens will be done on simple 2D tissue such as RPE monolayer cultures as demonstrated by Ferrer et al 2014. Once primary hits have been identified, lead optimization will be done using complex 3D tissues such as the OBRB.

22.5.2 Microfluidics

As discussed previously, another possible avenue for testing of ocular diseases lies in non-traditional culture of ocular cells. Microfluidic culture devices show promise in this regard due to their ability to have multiple chambers separated by porous membranes (Huh et al., 2010, 2011). Thus, reconstitution of multi-laminar, almost three-dimensional (Li et al., 2012) tissues is possible. Further, linking multiple chips together could begin to simulate system level complexity if each chip contained different tissues/organs. This idea has spurred the creation of the "human-on-a-chip" project (Marx et al., 2012; Sonntag et al., 2010) along with a variety of "organ-on-a-chip" devices (Huh et al., 2010, 2011). The aim of these projects is to bridge the divide between typical *in vitro* culture of cells and the *in vivo* systemic level response of animal testing using microfluidic chips. In theory, this system, in combination with iPSCs, will allow researchers to more accurately predict drug effects on individual humans by using patient specific, iPSC derived, cells differentiated into a variety of organs connected together in a similar fashion to how they would be connected in the human body. In this design each chip would represent the smallest functional unit of an organ, and because of their small size, and the parallelizability of microfluidic chips, this allows researchers to scale up the

system to apply to multiple patients or to have multiple replicates from a single patient simultaneously. Parallelization allows for the possibility of developing a more functional high throughput platform for evaluating drug efficacy and safety while simultaneously producing more clinically relevant results by using patient cells directly.

With multi-chamber microfluidic chip technology recreation of the BRB with endothelial cells and RPE on either side of a permeable membrane is possible. With this model such diseases as drug induced macular edema, which is due to disrupted RPE driven fluid transport across the OBRB, or genetic diseases such as BVMD, in which fluid transport across the OBRB is diminished, will be modeled in a physiologically accurate setting. Figure 22.2 (Plate 24) shows an example of how this multi-chamber microfluidic culture system might appear.

Equally important to the information gleaned from the OBRB/RPE microfluidic disease model is the fact that this model can then be added to other organs-on-a-chip such as the liver or kidney and the effect that drugs that are meant to treat RDDs have on these organs can also be assessed. Currently no microfluidic chips have been developed with ocular tissues such as the OBRB/RPE or RPE/photoreceptors. However, the system is ripe for development and with the advent of ever more efficient and stable differentiation protocols for RPE from iPSCs (Brandl et al., 2014; Nguyen et al., 2015a) the development of "eye-on-chip" systems is on the horizon.

Microfluidic devices provide a hope for testing potential novel therapeutic drugs, but as with other systems, there are several advantages and several limitations to most current microfluidic systems that stem from the choice of material used to make these chips. Microfluidic chips are typically made from polydimethylsiloxane (PDMS). Advantages of PDMS include: (1) PDMS is transparent to light from 240 to 1100 nm, thus optical detection methods from UV to IR are not obstructed so that imaging of cells stained with almost any fluorescent markers is possible; (2) nanoscale features down to ~400 nm are easily possible by pouring uncured PDMS into molds and then either thermally or UV crosslinking the PDMS (Mukhopadhyay, 2007); (3) PDMS is elastically deformable and thus it is hard to break and can be used to create valves; (4) PDMS is inexpensive, consistent material, and permeable to oxygen and CO_2; (5) PDMS can form covalent bonds with other PDMS surface or with any similarly oxidized surface. This removes the need for glues and also makes sealing multi-chamber chips dramatically easier (Berthier et al., 2012).

However, there are several limitations to PDMS that may detract its use. First, PDMS absorbs hydrophobic molecules and proteins (Toepke and Beebe, 2006). Since many drugs are lipophilic and most steroid hormones are hydrophobic, studies become difficult when drugs or hormones with these properties are involved (Griffith et al., 2014). In addition, PDMS allows oxygen, water, and CO_2 to diffuse through it affecting osmolality of solutions to sometimes harmful levels (Heo et al., 2007). PDMS is also mechanically deformable and non-conductive, which hinders its use for applications requiring large mechanical forces and electrical recordings. Lastly, un-crosslinked PDMS can leach into cells and can have detrimental effects on cells (Regehr et al., 2009).

Two opposing views have been suggested to address some of the limitations of PDMS: modify the structure of PDMS to overcome some of these hurdles or utilize new materials for microfluidic chips. Each of these approaches has pros and cons and neither should be considered as better than the other. As the pros and cons list previously shows, many traits that can be seen as desirable in one setting are undesirable in another (oxygen diffusion, for example). There are trade-offs for any material used and so a diverse, validated, material space for microfluidic chips where a researcher could select a material based off of desired experimental parameters is the ultimate goal in the field.

Finally, microfluidic chips do not truly recapitulate organ scale three-dimensional structure. This is due to their inherent small size and because manufacturing complexity increases dramatically as size increases. Currently, it is unclear whether true organ level function can be represented by such a small volume and thus,

until these models have been perfected, their actual utility for systemic drug performance is unknown. However, initial research appears promising and even if they are not able to be 100% predictive of system level, patient specific, response of drugs – microfluidic chips offer an additional method to validate drugs prior to human testing. This added level of scrutiny will undoubtedly lead to fewer false positives and more clinical success for drugs entering clinical trials.

22.6 Conclusion and Future Directions

iPSC technology has provided hope that more drugs that are relevant and safe for human use can be quickly discovered and brought into clinical use. We suggest the following phases for how iPSC technology can completely revolutionize the drug discovery process. The first phase of iPSC-based drug discovery involves disease modeling using patient-specific cells. Multiple different disease models in ocular and non-ocular disciplines have been set up using patient-specific iPSCs. This is the dawn of new era for truly personalized medicine. It has led to testing of several hypothesis-driven drugs against targeted disease-causing pathways. It is yet to be seen if any of these drugs can be commercialized for patient use. The second phase of iPSC-based drug discovery involves performing large-scale drug screens using 2D cell cultures. Several proof of principle HTP assays have been developed and tested, both in ocular and non-ocular fields. Primary hits identified using these large-scale HTP screens will feed the future drug discovery pipeline. The third phase of iPSC-based drug discovery involves the use of complex 3D-tissue that will aid in lead optimization and in confirming drug safety. Development of 3D-tissues using either developmental biology or tissue engineering has already begun, but this field needs to mature and derived tissues need to be functionally validated before they are routinely used for drug testing. In the fourth phase, iPSCs will be used for population based drug discovery. This phase will combine efforts from the first three phases and drug effects on a large patient population will be tested in a dish. Drugs that target a certain genetic group will be identified ahead of clinical trials. Several international efforts have begun generating large banks of patient-specific iPSCs for this purpose. Ultimately, it is hoped that HTP assays, complemented by iPSC technology and tissue engineering, will greatly enhance the discovery of cheaper and more effective drugs that are relevant at both individual and population levels.

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23

Important Considerations in the Therapeutic Application of Stem Cells in Bone Healing and Regeneration

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23.1 Introduction

The musculoskeletal system, primarily composed of the skeletal bones, muscles, cartilage, tendons, ligaments, and joints, is a collection of organs that provide the form, support, stability, and movement to the body. Since, the vital organs and tissues all encompass the musculoskeletal system, the musculoskeletal disorders or injuries can lead to the impairment or lack of function of any of these tissues, and hence, are a major health concern. Musculoskeletal disorders can consist of minor acute physical disabilities or major, complex, and chronic damage. These diseases can affect people and animals, both of which are a big health concern to veterinary and human medicine. The National Institute of Arthritis and Musculoskeletal diseases is a National institute with a scientific mission to improve public health by understanding, and providing strategies to repair the musculoskeletal system and the diseases that it affects.

Stem cell biology and regenerative medicine collectively constitute one of the most exciting areas of contemporary biomedical research. Specifically, adult mesenchymal stem cells (MSCs) are being used extensively in the research and the treatment of musculoskeletal diseases and disorders [1]. Adult MSCs are an attractive source of progenitor cells for the treatment of these diseases. The use of adult MSCs alleviates the ethical issues related to the use of embryonic stem cells in regenerative medicine.

Stem Cells in Toxicology and Medicine, First Edition. Edited by Saura C. Sahu.

23.2 Stem Cells, Progenitor Cells, Mesenchymal Stem Cells

The term stem cell appears in the scientific literature as early as 1868 described by the eminent German biologist, Ernst Haeckel. Research in stem cells and potentially regenerative medicine originated with the discovery of hematopoietic stem cells by McCulloch and Till in the 1960s [2]. Becker and colleagues demonstrated that injection of bone marrow cells into irradiated mice resulted in colonies, where the number was proportional to the number of bone marrow cells injected. They concluded that a given colony arose from a single cell. Further investigation revealed that these cells were capable of self-renewal, which is a primary characteristic of stem cells [3].

Stem cells begin to form in the very early stages of embryogenesis and the cells isolated from the inner cell mass in the blastocyst are referred to as the embryonic stem cells (ESCs). Alternatively, stem cells can be isolated from any adult tissue in the body and these are referred to as the adult mesenchymal stem cells (MSCs). These have been isolated from tissues including skeletal muscle [4], intestine [5,6], brain [7,8], dental pulp [9], skin [10], and most recently the hair follicle [10]. More specifically, MSCs have been isolated from the tissues that develop from the mesoderm germ layer, namely, bone marrow, fat/adipose tissue, cartilage, and muscle, and hence the name mesenchymal stem cells. Regardless of the source, both the ESCs and the MSCs are undifferentiated cells with the capability to proliferate limitlessly during the life of the organism, and to differentiate into specialized cells when they are located in a suitable environment. Due to the relative ease of harvest and lack of ethical concerns, MSCs are being used as a promising tool for research, clinical and commercial applications of cell transplantation, and cell-based therapy, including tissue engineering.

Mesenchymal stem cells refer to the non-hematopoietic (non-blood) fraction of cells. In order to maintain some uniformity in identifying MSCs, the International Society for Cellular Therapy (ISCT) has established three minimal criteria for outlining human MSCs [11].

Criteria 1: Adherence to plastic while maintained in standard culture conditions. When MSCs grow in culture, they appear fibroblastic, spindle-shaped in morphology, and as a cluster or a colony of cells. Each cluster is referred to as a colony and this property is often used as a measure of the colony forming unit [12,13].

Criteria 2: Expression of particular surface antigens. The recognition of cell surface markers, collectively referred to as the cluster-of-differentiation (CD) markers permit the identification of a particular cell population. When performing flow cytometry, approximately 95% of the MSC population should mainly express CD29, CD44, CD73, CD90, and CD105 surface antigens. The MSCs should also lack expression of the hematopoietic antigens CD45, CD34, CD14, CD11b, CD19, and human leukocyte antigen (HLA) class II. One of the drawbacks of these criteria is that the cell surface markers expressed on MSCs are not specific to MSCs only, but, can be expressed in other non-stem cell kinds, and hence, researchers must demonstrate the expression of more than one marker in a given population of cells to be classified as MSCs.

Criteria 3: Potential for multipotent mesenchymal differentiation. Since, one of the maximum vital traits of stem cells is its capacity to differentiate under optimal conditions, osteogenic, chondrogenic, and adipogenic differentiation profiles are considered the gold standard that is tested in culture prior to their use *in vivo* [14]. Besides the tri-lineage differentiation, MSCs have also been shown to undergo trans-differentiation, that is differentiate into non-mesodermal cell lineages, neural, endothelial, and hepatocyte-like [13,15–21].

It is important that for a proper identification of an MSC population of cells, all three criteria are taken into consideration simultaneously.

Because of the relative ease of the harvest and accessibility, the bone marrow and adipose or fat tissue-derived MSCs are considered as the gold standard [22–25]. Friedenstein and colleagues [26] demonstrated that a population of mononuclear cells isolated from the bone marrow were clonogenic and adhered to the

polystyrene-treated tissue culture plastic. Subsequently, different groups have described subsets of cells isolated from the bone marrow with properties similar to the cells described previously. Since, then, the MSCs have been referred to as mesenchymal stem cells/marrow stromal cells [27,28], bone marrow stromal stem cells (BMSSC) [29], marrow-isolated adult multipotent inducible cells (MIAMI) [30], and mesenchymal adult stem cells (MACs) [31].

Zuk and co-workers [32] had been the primary to describe the presence of MSCs in human white fat tissue. The subcutaneous adipose tissue is considered to be an abundant source of MSCs compared to the visceral fat [33–35]. The visceral fat has fewer progenitor cells relative to the subQ fat and also MSCs derived from visceral fat exhibit lower proliferation and differentiation capacity [36,37]. Typically, the adipose tissue is harvested and subjected to enzymatic digestion and the stromal vascular fraction (SVF), containing the macrophages, endothelial cells, T regulatory cells, and comparatively, a much higher percentage of MSCs is obtained [14,38].

Even though both the bone marrow and adipose tissue are considered as the preferred sources of MSCs, cells derived from the adipose tissue are preferred. Bone marrow harvest can potentially be associated with the harvest site morbidity, and pain. Some of the striking differences in MSCs between the two sources and of relevance to our research, include, donor age and variation in their capacities for osteogenic differentiation. Bone marrow aspirates contain only small proportion of MSCs and thus, the osteogenic differentiation capacity decrease with the age of the donors [39,40]. While the adipose tissue is abundant, relatively easy to access from the body and the cells number do not decrease with the age [32]. Although MSCs from different tissues show similar phenotypic traits, it's not clear if these are the identical MSCs as they certainly display different propensities in proliferation and differentiation [1].

In summary, the MSCs from both sources are multipotent, are capable of expansion *in vitro*, and can be cryopreserved for long periods without significant loss in proliferation and differentiation capacity [41].

Even though MSCs are being used extensively in basic and clinical research, a lot of researchers are still trying to investigate their role(s) in tissue healing and regeneration. The following are some of the proposed roles that MSCs might play at the site of the injury:

1. Anti-Inflammatory: MSCs modulate the expression of pro- and anti-inflammatory cytokines [42–44].
2. Immune response: MSCs modulate immune response by inhibiting mitogens and alloantigens, which leads to inhibition of lymphocyte activation and proliferation [45].
3. Antibiotic role: MSCs modulate bacterial infection through secretion of the antimicrobial peptide, LL-37 [46].
4. Neovascularization: MSCs play an active role in the procedures involved in the formation, stabilization, and maturation of newly formed vessels [47,48].
5. Recruit the native MSCs and progenitor cells: MSCs motivate the resident progenitor cells and enhance their proliferation and differentiation [49–51].
6. Autocrine effect: MSCs themselves contribute to the remodeling of the tissue through differentiating into the injured tissue. It has been shown that the injected stem cells recruit to the site of the injury and differentiate to replace the damaged tissues [52,53].
7. Paracrine effect: It has been proposed by number of the researchers that the stem cells releasing paracrine factors and produce local signaling molecules that may not only help in regeneration of the tissues but also limit the tissue apoptosis [54–56].

Biochemical and chemical signals are used to trigger or to promote the commitment of MSCs into various lineages. Lineage-specific differentiation induction factors can be of protein and/or chemical origin. Biological factors have been demonstrated to induce the differentiation of stem cells, *in vitro* and *in vivo*. For example, bone morphogenetic proteins (BMPs), such as BMP-2 and BMP-7, are the most potent growth

factors required for an efficient osteogenesis of MSCs and enhancing bone formation [57]; transforming growth factors β1 and 3 (TGF-β1 and TGF-β3) are routinely used to enhance the chondrogenic differentiation of the MSCs [58]; brain-derived neurotrophic factor (BDGF), platelet-derived growth factor (PDGF), glial cell line-derived neurotrophic factor (GDNF) [59], basic fibroblast growth factor (bFGF) [60], and the epidermal growth factor (EGF) can be utilized to promote neural trans-differentiation of MSCs [61]; hepatocyte growth factor (HGF), EGF, TGF, and insulin-like growth factor induced transdifferentiation of MSCs into hepatocytes [15–17]. Additionally, some of the chemicals frequently used in the lineage-specific differentiation of stem cells *in vitro*, include dexamethasone (DEX), ascorbic acid and β-glycerophosphate as osteogenic inducers [62]; retinoic acid (RA) for neural differentiation of ESCs [63] and β-mercaptoethanol (BME) is utilized for neural trans-differentiation of MSCs [61]; and DEX, RA, sodium butyrate, and nicotinamide (NTA) act as inducers for hepatic trans-differentiation of MSCs [15–17].

In the last decade or so, it has been recognized that in regenerative medicine, mere use of MSCs or progenitor cells is not sufficient for an efficient treatment or therapy. An effective regenerative therapy includes the identification and characterization of MSCs, identification and synthesis of biomaterials that can be used as scaffolds, either alone or in combination with MSCs; understanding the microenvironment of the diseased or the injured tissue, and finally, the combination of all three factors. This is also referred to as tissue engineering and encompasses all of these aspects.

23.3 Scaffolds

Simple factures in our bones usually heals spontaneously, but large areas of damage will take longer time to heal may require scaffold to regenerate new and robust bone. The required scaffolds have to meet rather unique and complex requirements, dependent upon the type as well as the location of the osseous tissue to be regenerated. The most critical need is for the materials to be biocompatible, allow an excellent cellular proliferation and provide the environment for the new tissue to form. In large-size bone defects, scaffolds are necessary to maintain the structural integrity of the damaged area. Those scaffolds ought to provide enough preliminary mechanical strength and stiffness to substitute for the loss of mechanical function of the diseased or damaged tissue. The scaffolds may not be mechanically equivalent to the healthy tissue, but their stiffness and strength should be sufficient enough to at least support and transmit forces to the host tissue site [64]. In bone tissue engineering, it is desirable to develop a biomaterial or a scaffold that can chemically and structurally mimic the native extracellular matrix of the natural bone, while providing some of the characteristics that the missing tissue would regenerate.

An optimal scaffold suitable for a bone defect must fulfil certain criteria [65] (Figure 23.1/Plate 25). First, it should be biocompatible, in which cells must adhere, and proliferate in the scaffold; second, it should be porous, such that the cells can penetrate the scaffold and there is adequate diffusion of nutrients to the cells; third, the scaffold should have mechanical properties consistent with the anatomical site into which it is to be implanted; and fourth, the scaffold should be biodegradable. With time, the scaffold should degrade and be replaced by body's own cells.

Ideally, researchers aim to develop composite scaffolds with nanofiber ultrastructure that aid in the establishment of the extracellular matrix of natural bone. In the last 50 years or so, biodegradable materials with specific hydrophilic/hydrophobic domains and amino acids, peptides, and/or functionalized proteins have been synthesized to mimic the biochemistry of living tissues and make these biomaterials responsive to tissue remodeling [66–68]. Engineering solutions were offered to obtain composite materials with tuned physicochemical properties and biomimicry of bone architecture [69]. An ideal biomaterial scaffold developed for bone regeneration should allow the remodeling of the new bone and assume the mechanical support. The scaffold should support the area undergoing reconstruction by providing the optimal mechanical strength, it should

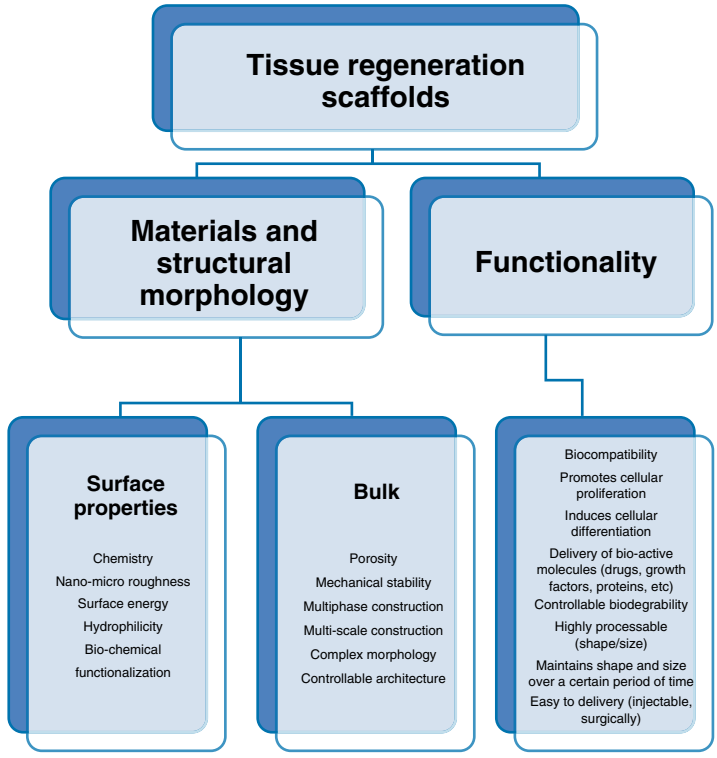

Figure 23.1 ***(Plate 25)*** *The complex set of requirements that a successful candidate for a tissue regeneration scaffold should include. (See insert for color representation of the figure.)*

stimulate new bone formation in the defect region and then should degrade without causing any remarkable inflammatory response [70]. A more detailed discussion of the scaffold materials as well as characteristics needed for bone regeneration, has been discussed recently by our group (Kadam *et al.* Drug Metabolism Reviews 2015, Accepted for publication).

23.3.1 Graphene

In the past two decades, a large number of materials have been proposed as candidates with high potential for tissue regeneration. Given the complex architecture of the bone tissue, with complex architecture both at the nano and micro levels, it is expected that nanomaterials will play a major role in this aspect. Nanotechnology has made significant progress and has generated the next generation of biomaterials to use in bone regeneration. Out of the plethora of nanomaterials proposed for bone regeneration, we focus on the carbonaceous ones given their high stability, interesting morphology, ease to integrate into two- and three-dimensional scaffolds and ability to functionalize their surface with a variety of functionalities and bioactive molecules [71–75].

Graphene family of nanomaterials is one such type of biomaterials presented in 2004 by Nobel Laureates, Geim and Novoselov [76]. Graphene is inert, made up of carbon atoms with unique electrical, mechanical and thermal properties making it a very good biomaterial. Due to its physical properties and inert nature, graphene is used in various medical devices, implants, sensors and of relevance to our study, in bone tissue engineering, given its ability to support cellular proliferation/differentiation, delivery of bio-medical molecules.

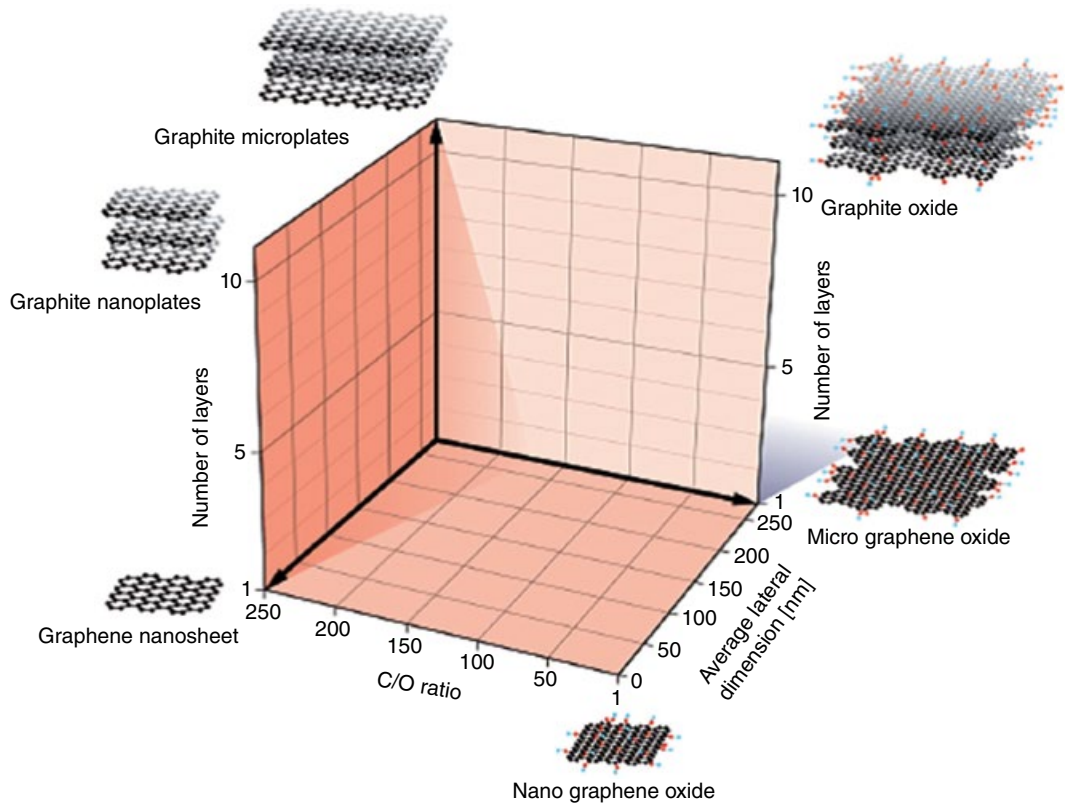

Figure 23.2 (Plate 26) *Schematic representing the various species of graphene structures. Adapted from Wick et al. [81], with permission of Wiley. (See insert for color representation of the figure.)*

Specifically, carbon nanotubes (single and multiwalled) and graphene have been recognized as the smart biomaterials with osteoinductive properties [77–80]. Graphene-related materials exist in layers (mono or multilayered) of sp^2-hybridized carbon atoms. These materials can be chemically modified into graphene oxide (GO) where a majority of the carbon atoms exist in a sp^3-hybridization with extensive oxygen functionalities (e.g., carboxylic, hydroxyl, epoxy, etc.). Other analogous forms are reduced graphene oxide (rGO) and oxygen-functionalized graphene (oxidized graphene) where much of the sp^2-hybridization remains and a lesser amount of oxygen functional groups are present on the surface of the graphene lattice (Figure 23.2/ Plate 26). Each of the aforementioned types of graphene nanomaterials can be produced by a variety of techniques and each may have a specific role in biomedicine.

Oxidized graphene is amphiphilic, can be dispersed in water and tissue culture media, and thus can be used relatively easily. Oxidized graphene is the preferred form of graphene in biomedical applications, specifically, bone tissue engineering [82]. It consists of a single layer of graphene sheets with carboxyl, epoxyl, and hydroxyl groups, providing an amphiphilic sheet of structure which can react with hydrophobic molecules [74]. Furthermore, oxidized graphene can be functionalized with a number of water soluble biocompatible polymers like polyethylene glycol (PEG) [83–86], polyethyleneimine (PEI) [87,88], polyvinylimidazole (PVI), and polyLlysine (PLL) [89], resulting in nanocomposites ideal for tissue engineering. The combination of graphene-based composites with nanofibrous polymers, PLL or PEG result in structures that mimic the extracellular matrix for bone regeneration. All these data suggest

that graphene-based nanomaterials can be synthesized in many shapes and forms with varied chemistries, which can significantly impact their application in regenerative medicine.

The response of immortalized cell lines or the primary cultures of stem cell populations has been evaluated on graphene-based biomaterials, by assessing cell adherence, proliferation and function by many researchers [78,79,90–92]. Graphene oxide has been covalently linked to hydrogels with improved cell adhesion, differentiation, proliferation of mouse pre-osteoblast MC3T3-E1 cells and human, mice and goat mesenchymal stem cells derived from bone marrow or fat [77,92–95]. Surface roughness and the oxygen content of graphene nanomaterials are believed to influence the adsorption of serum proteins which enhances the cellular behavior on graphitic surfaces. It has also been suggested that the rough surface absorbed the key proteins (integrins) expressed in the osteoblasts and hence improved cell adhesion, anchorage, and osteogenic differentiation [96]. Lee *et al*., reported that the noncovalent binding capabilities of graphene allows them to act as a foundation where the osteogenic inducers can concentrate and induce MSCs toward the osteogenic lineage [79]. A majority of these studies report the effect of 2D and 3D graphene-based materials on the adhesion and osteogenic and neural differentiation of MSCs. There are some studies describing that graphene lacks cytotoxicity, proving the biocompatibility and its ability to induct MSCs for bone formation *in vivo* [97–99]. To date, there is no report describing the efficacy of graphene or its derivatives in bone regeneration in a controlled animal model. As a result, very little is known about the cellular mechanisms and signaling pathways that are triggered when cells adhere and proliferate on graphitic surfaces. Despite many unanswered questions, the graphene family of nanomaterials definitely present themselves as an attractive biomaterial that can be used in bone tissue engineering applications. Future studies are required to understand these mechanisms and the signaling pathways. Preclinical studies and controlled animal models will prove helpful in the progress of graphene-based nanomaterials for clinical use.

23.4 Animal Models in Bone Healing and Regeneration

Numerous animal models have been developed through the years to verify the practicability of research tactics and stimulate clinical situations mimic the natural ones. Animal models allow the evaluation of stem cells alone or in combination with biomaterials over potentially long time durations and in different tissue qualities [100].

Various species have been utilized as animal models of bone defects, including mice, rats, rabbits, dogs, pigs, sheep, and goats, yet a significant part of the exploration has centered around rodent models due to reproducibility, high throughput, and economic and time considerations [100,101]. An ideal animal model should be highly reproducible, relevant to the clinical situation under consideration, suitable to use for the assessment of multiple types of materials or strategies, and offer little morbidity and mortality to the animal prior to the planned experimental endpoint [102]. Literature review shows that tremendous amount of research in regenerative medicine is being done *in vitro*. Comparatively, there are very few *in vivo* reports. As a result, it is not known whether the stem cells with or without any biomaterial behaves similarly *in vivo*. Hence, animal models that mimic naturally occurring disease(s) are very crucial.

23.4.1 Bone Regeneration Models

To evaluate the bone regeneration capacity of stem cells, scaffolds, or a combination of both, the chosen animal model can be hold the disease spontaneously or can be induced either chemically or surgically.

Ectopic (Heterotopic) bone model was evolved as an assay for osteoblast differentiation and stem cell characteristics of MSCs *in vivo* by implanting the sample in a place other than the bony compartment. In this model, bone formation is usually induced at an intramuscular or subcutaneous anatomical site by implanting the cells or scaffold in soft tissues [103–106].

For testing the bone formation capacity of scaffolds, cells, or both, a more specific defect model is required to test the capability to regain or restore the normal anatomy or normal function of the bone. Described next is a compilation of the bone defect models that have been used either for testing the effect of chemical drugs or of stem cells alone or in conjunction with biomaterials/scaffolds.

Osteoporosis is one of the common diseases that affect the bone. The osteoporosis model can be achieved by hormonal disturbance (ovariectomy, orchidectomy, hypophysectomy, parathyroidectomy), immobilization, and dietary manipulations [107]. Immature or young animals are appropriate animal models to study endocrine, environmental or nutritional osteoporosis, whereas, mature and aged animals are appropriate for research conducted on postmenopausal and immobilization osteoporosis [108]. Spontaneous animal models (those animals exist in nature with similarity to certain condition or disease) can also be used. For example, *MRL/lpr* is a well-known mouse model for human systemic lupus erythematosus-like disorders [109]. Since, the *MRL/lpr* mice exhibit a severe reduction of the trabecular bone [110], this model is appropriate to study secondary osteoporosis.

Osteonecrosis or bone death can result from poor blood supply to an area of the bone. Osteonecrosis can be induced by the administration of steroids or bisphosphonates [111]. Osteosclerosis is defined as abnormal hardening of bone which resulted in high skeletal density. This model has been shown to be induced by the overexpression of thrombopoietin by retroviral-mediated transduction of bone marrow MSCs [112]. Osteonecrosis has also been shown to be induced by infection with Reilly-Finkel-Biskis murine leukemia virus (MuLV). The MuLV has been proven to induce lymphomas, osteosclerosis, and osteomas in susceptible strains of mice [113]. Reid *et al* assumed that the mechanism of action is probably through over-suppression of bone turnover [114]. Also its accumulation in bone to a level being toxic to the oral epithelium leads to failure of healing of soft tissue lesions. Noggin is a bone morphogenetic protein inhibitor and it has been shown that the overproduction of noggin during biological aging inhibits osteoblast differentiation and bone formation [115].

Otosclerosis is a genetic disease in which the bone loses its elasticity, which ultimately leads to functional disturbance. The *Lp/J* inbred mouse has been demonstrated to develop abnormal bony lesions of the middle ear, and hence, is used as a model for human otosclerosis [116].

Bone in the sagittal section has two cortices. The cortical defect is known as removal of a defined area of the bone cortex from one of these cortices, in which the depth of the defect is defined by perforating the bone marrow cavity, while the size of the defect can be controlled by the size of the burr used in drilling the cortex. Cortical defect is a simple weight bearing model to test the effect of any cell-based therapy [117]. It can also be used to test the integration capacity, by using a screw coated with the material of interest [118]. A new double screw hole cortical defect bone model has been described to test the filling capacity and quality of the new bone formed in a mandibular defect [119].

In a segmental defect model, a fracture is created in a long weight-bearing bone by removing a complete segment of the bone. The surgical procedure to remove bone segment is followed by implantation of the test material (cells and/or scaffolds). The disadvantage of this method is that fracture fixation is required for bone fragment stabilization, which may add another variable to consider when the outcome is assessed. As a result, this model is relatively difficult and challenging to control in large animals, and hence, may require additional groups of animals and longer time points that can prove expensive and inconclusive. To overcome this variability, it is preferred to choose a nonbearing bone, such as the ulna.

Maxillofacial bone defect model is a commonly used research model created either in the frontal bone or the mandible. This bone model is advantageous over the long bone models in that the cortical and trabecular bone material is bilateral, non-weight bearing, and the defect can have bilateral symmetry (frontal bone and rami). The models of oral and maxillofacial bone defects include the calvarial [120,121], the maxillary/alveolar cleft [122–126] and the mandibular [127–130] defect. The only disadvantage this model has is that the assessment is not straight forward and the bone healing cannot be evaluated by visual inspection for instance, walking, in case of long bone defects [102].

Due to the relative ease of access, most of the animal models for bone tissue engineering are based on the long and the maxillofacial bones. However, these structures are quite different from the cancellous bone of the vertebrae, and hence, spine defect models may not be the best bone models but are relevant and of major concern to the health and well-being. The spine defect models are usually conducted on one of the three main parts of the vertebrae; the body [131,132], the vertebral arch [133] or the transverse processes [134]. In a spine defect model, a large volume of bone tissue from the vertebra is lost, resulting in a deficiency of the bone marrow-containing osteogenic progenitor cells. Since, bone marrow-derived MSCs function in an autocrine and a paracrine manner, this deficiency can affect the process of bone regeneration [135]. A combination of mesenchymal stem cells with a polysulfate-based scaffold has been recently described in a spine defect model in sheep [136].

Most of the studies conducted at the level of preclinical models (Table 23.1) have been carried out using naïve, undifferentiated MSCs derived from bone marrow [97,110,132,140,145,148,164,165,167] and adipose tissue [144,158,160,163]. There are some studies, however, in which the efficacy of MSCs has been assessed after osteogenic differentiation [131,133,143,146,156,161]. The osteogenic potential of other sources of adult mesenchymal stem cells including, peripheral blood [145], alveolar-bone [166], umbilical cord [122], periodontal ligament [157], and urine [150] has also been tested for bone healing *in vivo*.

Additionally, many studies are reported where a scaffold has been used to deliver stem cells and to hold the cells at the site of the defect. Some of the commonly used scaffolds are poly lactic-co-glycolic acid (PLGA) [131,139,151,156], hydroxyapatite [119,132,155,163], chitosan [130,157,168,169], platelet-rich plasma gel (PRP) [158,159,161], and ceramic [164–166]. In some studies, researchers have combined two or three scaffolds with or without cells, which can further make the functional assessment technically very challenging if appropriate treatment groups of animals are not included. In some studies, MSCs have been delivered systemically [110,137] or percutaneously at the site of the defect after the inflammatory response of the surgery subsided [167]. There are not many reports of using these strategies to deliver MSCs, since, these are based on the fact that MSCs will be attracted to and "home" to the site of injury without any scaffold.

23.4.2 Clinical Trials in Bone Regeneration

Although strong efforts have been made over the last decade to introduce stem cell and tissue engineering treatment strategies to the treatment of musculoskeletal diseases or injuries, only few clinical applications are currently available. Using some of the *in vitro* and preclinical *in vivo* studies, clinical trials using MSCs with or without scaffolds have been initiated and some of this research has been published. Researchers have used undifferentiated or differentiated BMMSCs and adipose or fat – derived MSCs in bone regeneration. Furthermore, undifferentiated or differentiated lineages of both types of MSCs have been used alone or can be combined with biomaterials and implanted as scaffolds.

Jager and colleagues, reported that when 10 patients with bone deficiencies were treated with MSCs and the bone marrow aspirates, the aspirate showed similar results compared to that of MSCs, suggesting that the local application of bone aspirate may be a promising alternative to autogenous bone grafting, or MSCs. Using the bone aspirate without any processing may help reduce donor site morbidity and save time [170]. Quarto *et al* [171] implanted porous bio-ceramic scaffold in combination with osteoprogenitor cells derived from bone marrow in three patients with segmental defects (ranging from 4–7 cm) and substantial improvement was observed. Also, Morishita *et al*. used the osteogenic differentiated BMMSCs mixed with hydroxyapatite (HA) ceramics to form bone matrix to fill the bone cavity after tumor curettage [172] and observed immediate healing. Serial plain radiographs and computed tomography images were used to evaluate the healing. No adverse reactions either due to the differentiated cells or the ceramics were noted.

Multiple reports have been published describing the treatment of nonunion and delayed union using stem cell therapy. The first cell-based strategy used for repair of bone nonunion was autologous bone marrow

concentrate harvested from the iliac crest and immediately transplanted to injured sites for skeletal repair in the same patient [173]. Twenty-eight patients with delayed union and three with nonunion of fracture of the long bones were treated with bone marrow injection which resulted in union in 23 cases with an average time of 12 weeks [174]. In yet another study, 49 patients with tibial nonunion were treated with bone marrow aspirates with demineralized bone matrix (DBM) and/or recombinant human bone morphogenic protein-2 (rhBMP-2). The results showed that the use of rhBMP-2 was associated with a lower healing rate compared to DBM alone [175]. One patient with a long-standing tibial nonunion defect was treated with a combination of calcium sulfate and 5 million tissue culture expanded BMMSCs. This patient had undergone six previous surgical procedures without any positive outcome. The nonunion was healed in 2 months post treatment [176]. One of the most complicated nonunion cases reported is the one associated with diabetes. Hernigou *et al*, demonstrated that there was 82.1 % significant increase in healing when 86 diabetic patients with ankle non-unions were treated with BMMSCs. Percutaneous technique was used to implant BMMSCs at the site of the defect. Relatively, there was only a 62.3% healing in 86 age- and disease-matched control patients treated with the standard autograft [177]. In 20 patients suffering from distraction osteogenesis [178], the transplantation of bone marrow cells and PRP shortened the treatment period by accelerating new bone regeneration. The same group also reported percutaneous autologous bone-marrow grafting for the treatment of an atrophic tibial diaphyseal nonunion with significant improvement [179]. A retrospective study using the same strategy for treatment of nonunion in 45 patients reported that 69% of non-unions at the tibia and 63% at the femur were healed, but none were noticed in the three humeri cases [180]. The patients enrolled in this study were followed for over a period of 8 years.

A 5-year follow up of a potential controlled study reported by Gangli and colleagues showed that the implantation of BMMSCs demonstrated a significant reduction in pain, joint symptoms and the incidence of fractural stages in non-traumatic osteonecrosis of the femoral head [181]. Stage 3 of the bisphosphonate-related osteonecrosis of the jaw (BRONJ) in a 75-year-old woman was successfully treated with bone marrow concentrate in combination with PRP [182]. Complete recovery was observed in 30 months post therapy.

Osteonecrosis of the femoral head is another clinical condition that has been intervened using cell-based therapies with variable results. Fifty-three patients with early-stage osteonecrosis of the femoral head were treated with 2 million culture expanded BMMSCs. A randomly selected 47 patients were subjected to the standard core decompression treatment. Only 2 of the 53 BMMSC-treated hips progressed and underwent vascularized bone grafting, and the rest of the cases improved significantly [183]. Wang et al. reported another study wherein, 15 patients were treated with a combination of the heterogeneous population of bone-marrow mononuclear cells and autogenous bone grafting [184]. The follow-up period ranged from 9–36 months and the overall success rate was estimated to be 80%. Implantation of the autologous BMMC with a minimally invasive technique resulted in significant pain relief and halted the progression of early stages of osteonecrosis of the femoral head in sickle cell disease patients. Of the patients, 96.3% showed disease stabilization and satisfactory results [185]. A study comparing the efficiency of bone marrow aspirate and the bone marrow mononuclear cells was conducted in 40 patients and 2-year follow-up analyses showed that the implantation of bone marrow mononuclear cells improved the hip function relatively better than the unprocessed aspirate [186].

There is one study that reports the use of bone marrow mononuclear cells with a porous hydroxyapatite biomaterial in the treatment of osteonecrosis of the femoral head [187]. Thirty-five patients were treated with the mononuclear cells and biomaterial scaffold, whereas, seventeen patients were treated with cell-free porous hydroxyapatite alone as a control group. The clinical success rate in presence of stem cells was 75.4% significantly higher in the BMMCs group compared with the control group. A single-center randomized clinical trial of 24 patients with end-stage osteonecrosis of the femoral head were treated with culture expanded BMMSCs associated with porous tantalum rod implantation combined with vascularized iliac grafting [188]. The mean Harris hip score demonstrated that all the treated hips improved significantly from 38.74 ± 5.88 points to 77.23 ± 14.75 points (range 33–95).

Table 23.1 *Summary of some of the published data where cell-based therapies have been used in bone pre-clinical models*

Model	Site of defect	Animal	Cell type	Cell source
Osteoporotic	Secondary/systemic osteoporosis	MRL/lpr mouse	BMMSCs	Allogenic
		MRL/lpr mouse	hBMMSCs/exfoliated deciduous teeth	Xenogenic
	Segmental defect in the radius	Rabbit	Fetal BMSCs	Allogenic
	Calvarial defect	Rat	AdSCs (from osteoporotic and normal animals)	Allogenic
Osteonecrosis	Femoral head trephine bone defect	Dog	BMSCs	Autologous
	Steroid-associated osteonecrosis	Rabbit	BMMNCs Fresh, cryopreserved	Autologous
	Jaw Bisphosphonate-related osteonecrosis	Rat	AdSCs	Allogenic
Segmental defect	Ulna	Dog	ASdCs	Allogenic
		Rat	ASCs	Allogenic
		Rabbit	PBMSCs/PBMNCs/BMMSCs	Allogenic
	Femur	Goat	Osteo-differentiated BMSCs	Allogenic
		Dog	BMMSCs	Autologous
		Rat	BMSCs	Allogenic
		Mice	hBMSCs induced to form cartilage tissue	Xenogeneic
		Rat	Human urine derived Stem Cells	Xenogeneic
		Minipig	AdSCs (transduced)	Allogenic
	Tibia	Monkey	BMMSCs	Autologous
		Sheep	BMSCs	Autologous
		Rabbit	hESC-MSCs	Xenogeneic
Cortical defect	Tibia	Rabbit	Rabbit Blood-derived Stem Cells/ BMMSCs	Allogenic
		Rat	ASCs transfected with microRNA	Allogenic

Scaffold	Length of the study	Study outcome	Ref
PBS (systemic injection)	4, 11 weeks	Using BMMSCs improved the reconstruction of bone marrow osteoblastic niche	[110]
PBS (systemic injection)	4 weeks	BMMSCs can be a good therapy for secondary osteoporosis in systemic lupus erythematosus	[137]
Decalcified bone matrix	3 months	Allogenic fetal BMSC has a potential effect on repairing bone defects in an osteoporotic condition	[138]
PLGA	32 weeks	ASC based tissue constructs helps the repair of calvarial defects in normal rats while implantation of PLGA scaffold was more advantageous for defect regeneration in OVX rats	[139]
Micro-CT-based bone ceramic scaffold	30 weeks	BCP scaffold resulted in significantly high osteointegration and new bone formation	[140]
	12 weeks	BMMNCs had a significantly higher effect on the bone density and vascularization of newly formed bone than the control group	[141]
PRP	9 weeks	BRONJ was effectively prevented with ASC-based treatments. The PRP proved to have a synergistic effect to the AdSCs	[142]
serum-derived albumin	16 weeks	The construct had significantly high bone regeneration	[143]
demineralized bone matrix (DBM)	24 weeks	DBM construct in combination with the cells promoted bone regeneration	[144]
porous calcium phosphate resorbable substitute	12 weeks	PBMSCs/BMMSCs was significant than PBMNCs in combination with the scaffold	[145]
Coral	4, 8 months	The defect was ideally healed using this combination	[146]
Chitosan	1.5, 3 and 6 months	Chitosan as a bone scaffold seeded with MSCs enhanced the process of bone healing than using of Chitosan alone	[147]
PA/PDLLA and PDLLA scaffold	4, 8, and 12 weeks	BMSCs + PA/PDLLA resulted in higher bone formation than BMSCs + PDLLA	[148]
Engineered CT/HT formed by MSCs	4,8 weeks	A decellularized engineered tissue was capable to promote tissue regeneration	[149]
β-TCP	12 weeks	USC is a good cell source for bone tissue regeneration	[150]
apatite-coated PLGA scaffolds	12 weeks	The transduced AdSCs with the FLPo/Frt-based baculovirus resulted in significant advance for the bone tissue regeneration	[151]
β-TCP ceramic scaffold	12 weeks	Prevascularized MSCs/scaffold had a significantly higher new bone formation	[152]
cortical allograft	6, 10, 14 and 18 weeks	Remodeling rate of the allograft was significantly higher in the cell-treated animals	[153]
3D unwoven macroporous nanofibrous (MNF)	3 and 6 weeks	3DMNF scaffold had bony tissue formation around and inside the scaffold as well	[154]
hydroxyapatite/tricalcium phosphate	3 and 6 weeks	The composite showed sig. higher bone healing than control. There was non-sig difference between both cell type	[119]
Hydroxyapatite	12 weeks	Transfection of ASCs with miR-26a resulted in significantly higher osteogenic potency	[155]

(*Continued*)

Table 23.1 *(Continued)*

Model	Site of defect	Animal	Cell type	Cell source
Maxillofacial defect	Calvarium	Rabbit	Osteogenic differentiated Ad-MSCs	Autogenic
		Rat	Periodontal ligament stem cells (PDLSCs)	Xenogeneic
		Rat	AdSCs	Allogenic
		Rat	UCMSCs	Allogenic
	Maxillary at M1 level	Rat	Human ADSCs	Xenogeneic
	Mandible PM2-M1	Dog	BMMSCs	Autologous
	Mandible	Rat	Osteo-differentiated SVF	Allogenic
	Mandible Canine extraction	Goat	BMMSCs	Autologous
	alveolar cleft (maxillary)	Dog	ASCs	Allogenic
		Pig	UCMSCs	Autologous
Spine defect	Vertebral body L2	Rat	Human BMMSCs	Xenogeneic
	Vertebral body L5 and L6		Osteo diff. AdSCs	Allogenic
	vertebral arch L5,6	Rabbit	osteo differentiated BMMSCs	Autologous
	Transverse process L2	Goat	BMSCs	Autologous
Ectopic model	Subcutaneous	Mice	Rabbit Blood-derived Stem Cells/ BMMSCs	Xenogeneic
		Mice	Human BMMSC	Xenogeneic
		Mice	human alveolar-bone-derived mesenchymal stem cells	Xenogeneic
	Intramuscular	Mice	BMMSCs	Allogenic

Scaffold	Length of the study	Study outcome	Ref
Polyamide, Poly lactic-co-glycolic acid and decellularized amniotic membrane	2, 4 and 8Wks	All the scaffolds showed sig. bone healing than the control. Polyamide has the highest bone formation	[156]
nanohydroxyapatite-coated genipin-chitosan conjunction scaffold (HGCCS)	12 weeks	This combination showed promising results for bone tissue regeneration	[157]
PRP	4 and 8 weeks	AdSC/PRP composite improved the bone healing and suggested as for clinical treatment of cranial defects	[158]
PRP	3 and 8 weeks	PRP enhanced the efficacy of UC-MSCs in bone regeneration *in vivo*	[159]
Amniotic membrane	21 days	ADSCs and AM co-culture system could strongly increase periodontal bone regeneration	[160]
PRP	2, 4, 8 weeks	The PRP + BMMSCs elicit true bone regeneration	[161]
chitosan scaffold	8 weeks	chitosan scaffold with SVF aliquots resulted in significantly earlier regeneration of bone	[130]
3D PLG	10 days-1month	BMMSCs in combination with the PLG were successfully differentiating to give the 3 basic tissues required for periodontal tissue regeneration	[162]
hydroxyapatite/beta-tricalcium phosphate scaffold	15 and 60 days	In comparison to the autograft, stem cells + scaffold sign lower than the autograft	[163]
electrospun nanomicrofiber scaffolds (NMFS)	1 month	Cells + scaffold similar to cancellous bone in the healing resulted	[122]
hydroxyapatite bone scaffold (CEM-OSTETIC®)	8 weeks	Cells + scaffold sig. higher than scaffold alone and cells alone	[132]
PLGA scaffolds	2, 4, 8 weeks	AdSCs induced to osteo diff. using GDF5, was sig. higher in bone regeneration	[131]
β-TCP bioceramics	2,4, and 8 weeks	Significant higher than scaffold alone	[133]
four different porous ceramic scaffolds	9 weeks	BCP and TCP porous scaffolds seemed most positive when compared to the HA scaffolds	[164]
hydroxyapatite/tricalcium phosphate	3 and 6 weeks	Sig. higher than control. Non-Sig between both cell type	[119]
resorbable ceramic (CDHA)	4 and 8 weeks	CDHA non-sig. to ß-TCP	[165]
biphasic calcium phosphate ceramics and Bio-Oss	8 weeks	Healing patterns of the scaffolds with hABMSC could be influenced by the microenvironment on the surfaces of the scaffolds	[166]
Graphene-containing composite nanofibrous scaffolds Carbon nanomaterials (CNMs) Carbon nanotube (CNT)	2, 4, and 8 weeks	Graphene composite nanofibrous scaffolds exhibited the most grounded impact on osteogenesis induction	[96]

Two successive reports describing oral and maxillofacial bone defects were published by a Japanese group, where, in the first report the group used naïve, undifferentiated BMMSCs in combination with PRP [189], while in the second report [190] they used differentiated MSCs in combination with PRP into a periodontal defect. Radiographic assessments showed regeneration and healing and showed promise in periodontal tissue engineering. Adipose tissue-derived stem cells seeded with fibrin glue were used to treat a traumatic calvarial defect in one patient and the treatment showed new bone formation and nearly complete calvarial healing in the 3 months post therapy [191]. Lee and colleagues used osteogenically differentiated BMMSCs as well to repair 15-cm segmental defects in the mandible, with reasonable success [192]. Similarly, osteogenically differentiated BMMSCs seeded onto partly demineralized allogenic bone matrix were used to treat seven patients of alveolar cleft. New, engineered bone was formed in 3 months post-operation [193]. Thesleff and colleagues used β-tricalcium phosphate scaffold in combination with autologous adipose-derived stem cells for reconstruction of large cranial defects and revealed that the combination was promising for treatment of calvarial defects [194]. Recently, in 2014, Sandor and group used the same combination in 13 cases of cranio-maxillofacial hard-tissue defects and successful integration of the construct to the surrounding skeleton was observed in 10 of the 13 cases [195]. The same group, subsequently used BMP-2 in addition to the previously mentioned treatment to successfully reconstruct a 10-cm anterior mandibular ameloblastoma resection defect [196].

23.5 Conclusions and Future Directions

In this chapter, we have briefly described the recent advances in bone tissue engineering. We have presented reports to describe how a multitude of factors, including, the source of the cells, physical and chemical properties of biomaterials, defect or the type of the injury, mode of cell/scaffold application, can affect the outcomes. Although these reports are exciting, the major drawback is that the follow-ups have been limited and the quality and composition of the regenerated bone is uncertain at this point because of the lack of the control groups. Furthermore, many aspects of the physiological processes involved are just beginning to be recognized, and there is still much more to be learned regarding cell recruitment, differentiation and behavior. Although the studies described here are exciting, reports highlight some of the aspects that limit bone tissue engineering from becoming a routine in clinical practice.

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24

Stem Cells from Human Dental Tissue for Regenerative Medicine

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24.1 Introduction

The hallmark feature of stem cells is that they possess multipotency and self-renewability. Stem cells are most commonly derived from early embryos (embryonic stem cells, ESCs) and adult tissue (adult stem cells, ASCs). As a main source of ASCs, mesenchymal stem cells (MSCs) play an important role in research and medical therapy. MSCs are spindle-shaped cells with the potential for clonogenic proliferation and multilineage differentiation. They were initially reported as fibroblast-like cells that could be isolated from bone marrow via their adherence to plastic in culture and subsequently confirmed as a population of bone-marrow derived non-hematopoietic cells with a colony-forming unit [1]. MSCs can differentiate into all mesodermal lineages, which prompted the investigation into the role of MSCs in mediating tissue regeneration [2]. The capacity of MSCs differentiate into mesodermal [3], ectodermal [4], and endodermal [5] cell lineages has since been fully characterized and forms the basis for most current work on bone marrow-derived MSCs (BMMSCs). In 2006, the International Society for Cellular Therapy (ISCT) [6] proposed the minimal characterization criteria for human MSCs, including their propensity to adhere to plastic when maintained under standard culture conditions and their ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*. In addition, most (≥95%) MSCs positively express CD105 (endoglin), CD73 (ecto-5′-nucleotidase), and CD90 (Thy1) while negatively express (≤2%) CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR [6].

Since the discovery and characterization of BMMSCs, MSC-like populations from other tissues have been characterized based on the standard criteria established for BMMSCs [1–3, 6, 7]. Additionally, MSC populations

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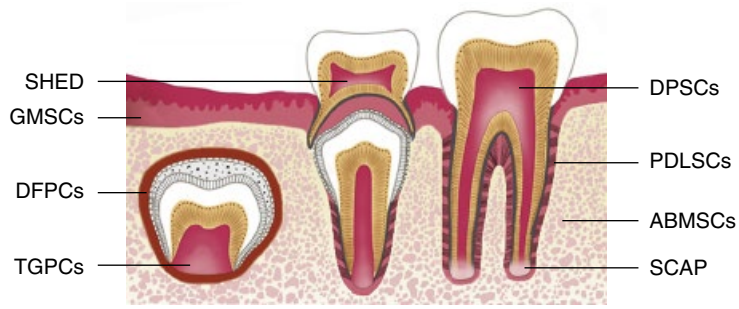

Figure 24.1 (Plate 27) *Schematic drawing illustrating sources of human dental tissue-derived MSCs. DPSCs: dental pulp stem cells; SHED: stem cells from exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; DFPCs: dental follicle progenitor cells; ABMSCs: alveolar bone-derived mesenchymal stem cells; SCAP: stem cells from the apical papilla; TGPCs: tooth germ progenitor cells; GMSCs: gingiva-derived MSCs. (See insert for color representation of the figure.)*

can be readily obtained from skeletal muscle [8] and a variety of other tissues, such as umbilical cord blood [9], synovium [10], the liver [11], adipose tissue [12], the lungs [13], amniotic fluid [14], tendons [15], placenta [16], skin [17], breast milk [18], and urine [19].

The search for MSCs in specific tissues led to the discovery of a distinctive population of MSCs from a variety of human dental tissues in previous decades. Up to now, eight unique populations of dental stem cells (DSCs) have been isolated and characterized. Postnatal dental pulp stem cells (DPSCs) were the first human dental stem cells to be identified from pulp tissue [20]. Gradually, other dental stem cell-like populations, such as stem cells from human exfoliated deciduous teeth (SHED) [21], periodontal ligament stem cells (PDLSCs) [22], dental follicle progenitor cells (DFPCs) [23], alveolar bone-derived MSCs (ABMSCs) [24], stem cells from apical papilla (SCAP) [25], tooth germ progenitor cells (TGPCs) [26] and gingival MSCs (GMSCs) [27], were also reported (Fig. 24.1/Plate 27).

This review includes preliminary data suggesting that these dental stem cells not only display self-renewal and multi-differentiation potential but also possess immunomodulatory functions and potent tissue regenerative properties (Fig. 24.2/Plate 28). A better understanding of the biological characteristics of DSCs is essential to investigate their potential for clinical application. Herein, we review many aspects of current investigations into various DSC populations [28].

24.2 Dental Stem Cells

24.2.1 Dental Pulp Stem Cells

DPSCs were first isolated by enzymatic digestion from adult human dental pulp tissues [20]. DPSCs show similar characteristics to BMMSCs [29], both populations express similar putative stem cell surface markers, such as CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146, and STRO-1, but not CD14, CD24, CD34, CD45, CD19, and HLA-DR [28, 30]. These cells show a higher proliferation rate than BMMSCs, they possess immunosuppressive properties, and they are prone to forming a dentin-pulp-like complex [29, 31].

Aside from their odontogenic potential, DPSCs are also capable of neurogenic, osteogenic, adipogenic, chondrogenic, and myogenic differentiaton [30, 32–35]. Recently, DPSCs exhibited the additional potential to differentiate into melanocytes, corneal epithelial cells, and hepatocyte-like cells (HLCs) *in vitro* [33, 36–38], and could even be used for derivation of induced pluripotent stem cells (iPSCs) [39]. More recently, a study reported the capability of DPSCs to differentiate to corneal stromal keratocytes *ex vivo* under induction [40].

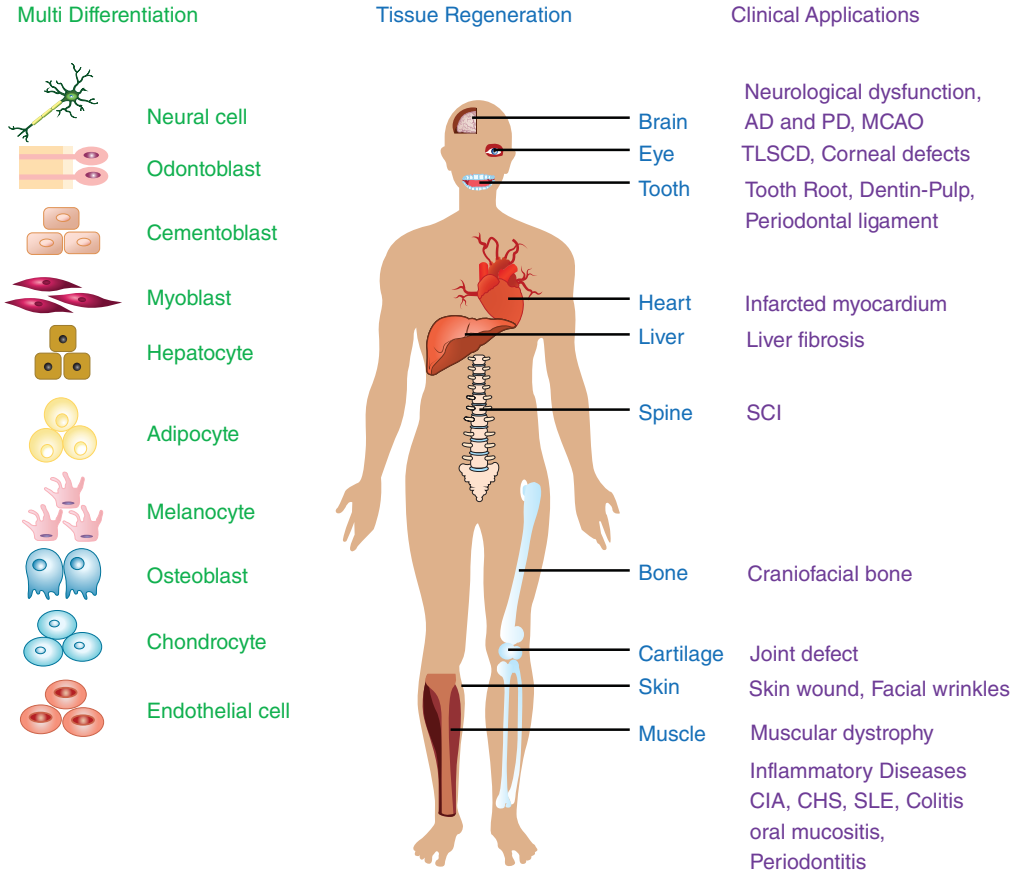

Figure 24.2 ***(Plate 28)*** *Multilineage differentiation capacity, tissue regeneration and potential clinical applications of human dental tissue-derived MSCs. AD: Alzheimer's dementia; PD: Parkinson's disease; MCAO: middle cerebral artery occlusion; TLSCD: total limbal stem cell deficiency; SCI: spinal cord injury; CIA: collagen-induced arthritis; CHS: contact hypersensitivity; SLE: systemic lupus erythematosus. (See insert for color representation of the figure.)*

Ex vivo expanded DPSCs can generate a dentin-pulp-like complex associated with vascularized pulp-like tissue and surrounded by a layer of odontoblast-like cells when transplanted into immunocompromised mice with hydroxyapatite/tricalcium phosphate (HA/TCP) as a carrier [20, 41]. Additionally, DPSCs can form mineralized nodules with a reparative dentin-like tissue on the surface of human dentin *in vivo* [41], and several scaffolds or carrier materials can be used to generate dentin-pulp-like structures, such as calcium phosphate scaffolds [42], polylactic acid [43], and hexafluoro-2-propanol silk [44]. Moreover, a bone-like tissue was formed in DPSC-transplanted samples *in vivo* with various scaffolds or carrier materials [45]. Reports have shown that DPSCs can differentiate into adipocyte-like cells, endotheliocytes, and myofibers and enhance angiogenesis *in vivo* [33, 46]. Studies also indicate that DPSCs survive in the central nervous system, express neuronal markers, and acquire neuronal morphology after transplantation into mesencephalon of chicken embryos [35]. After transplantation into the brain of newborn rats, DPSCs exhibit homing to cortical lesion sites indicating their potential to rescue or replace damaged neurons [47]. They are known to exert paracrine neuro-tropism through their vast expression of growth factors [48].

24.2.2 Stem Cells from Human Exfoliated Deciduous Teeth

A distinct population of clonogenic, highly proliferative postnatal stem cells can be isolated from the remnant pulp of exfoliated deciduous teeth and expanded *ex vivo*, thereby unexpectedly providing a unique and accessible tissue source of MSCs [21]. SHED are distinct from DPSCs due to their higher proliferation rate, increased cell population doublings rate, and ability to form sphere-like cell cluster. SHED display surface markers that conform to the minimal criterion for MSCs proposed by ISCT with DPSCs [49, 50], and they also were found to express embryonic stem (ES) cell markers Oct4, Nanog, stage-specific embryonic antigens (SSEA-3, SSEA-4), and tumor recognition antigens (TRA-1–60 and TRA-1–81) [21]. Cultured SHED also express the cell surface molecules STRO-1 and CD146, early MSC markers previously found to be present in BMMSCs, and DPSCs [21]. As neural crest cell-associated postnatal stem cells, SHED express a variety of neural and glial cell markers, including nestin, β III tubulin, GAD, NeuN, GFAP, NFM, CNPase, and Pax6, which may be related to the neural-crest cell origin of DPSCs, indicating a good neural cell differentiation potential [21, 51].

Similar to DPSCs, SHED are capable of differentiating into osteogenic, odontogenic, and adipogenic cells. Furthermore, SHED express a variety of neural cell markers, form sphere-like clusters, and form multicytoplasmic processes when cultured under neurogenic conditions [21]. The tendencies for myogenisis and chondrogenisis of SHED have been demonstrated [52]. Reports also support the observation that SHED can differentiate into endothelial cells when cultured on a dentin slice *in vitro* [53]. Under hepatic differentiation condition, SHED were shown to produce specific hepatic proteins and they acquired the morphological and functional characteristics of hepatocytes [54]. SHED can also be reprogrammed into iPSCs [39].

The neural developmental potential of SHED was studied by injecting SHED into the dentate gyrus of the hippocampus of immunocompromised mice. SHED could survive more than 10 days and continued to express neural markers, such as neurofilament M [21]. After *in vivo* transplantation into the intraperitoneal space, SHED were shown to undergo dense engraftment in various tissues and organs, including the liver, spleen, and kidney, indicating their potent differentiation plasticity [52]. SHED can repair critically sized calvarial defects in immunocompromised mice through substantial bone formation [55]. SHED induced new bone formation by recruiting host osteogenic cells *in vivo* even though they could not differentiate directly into osteoblasts. These findings imply that deciduous teeth may not only provide guidance for the eruption of permanent teeth, as is generally assumed, but they may also be involved in inducing bone formation during the eruption of permanent teeth [21]. *Ex vivo*-expanded SHED yielded odontoblasts that were directly associated with a dentin-like structure after transplantation into immunocompromised mice [21]. However, unlike DPSCs, they failed to reconstitute dentin-pulp-like complexes *in vivo* [20]. SHED seeded onto tooth slices/scaffolds were capable of differentiating into functional blood vessels that connected with the host vasculature and formed a dental pulp-like tissue and dentin after subcutaneous implantation into immunocompromised mice [56]. With a PEGylated fibrin carrier, SHED rendered a vascularized soft connective tissue similar to dental pulp after *in vivo* transplantation [57].

24.2.3 Periodontal Ligament Stem Cells

The periodontal ligament (PDL) is a soft connective tissue embedded between the cementum and the alveolar bone socket. Early evidence showed that PDL not only plays an important role in supporting teeth, but it also contributes to tooth nutrition, homeostasis, and regeneration of periodontal tissues [58].

Explant cultures or enzyme digestion treatment of the PDL released a population of PDLSCs; postnatal multipotent stem cells that could be readily expanded *in vitro* to generate a cementum/PDL-like complex. Additionally, PDLSCs show more population doublings in culture [22] and express STRO-1 and other cell

surface markers that are also present on DPSCs. PDLSCs express a higher level of the tendon-specific transcription factor, scleraxis than do DPSCs, suggesting that PDLSCs might form a unique population of postnatal MSCs [22, 59]. PDLSC populations express a heterogeneous assortment of makers associated with dentin, bone, smooth muscle, neural tissue, and formation of calcified nodules [60]. Similar to the other dental stem cells described previously, PDLSCs have the ability to differentiate into osteogenic, adipogenic, and chondrogenic cells under defined culture conditions [61]. Trans-differentiation of PDLSCs into pancreatic cell lineage was also evidenced by the formation of pancreatic islet-like clusters, insulin secretion, and pancreatic marker expression [62]. Myotubular-like structures, indicative of skeletal myogenic differentiation, have been generated from PDLSCs after 5-azacytidine induction [63]. Further *in vitro* studies showed that PDLSCs have the capacity to become retinal progenitors exhibiting $Pax6^{nuclear}$ Rx^{+} phenotype, and generated mixed types of retinal neurons with a predominant photoreceptor phenotype expressing both rhodopsin and Nrl after prolonged induction, these exciting findings show the potential of differentiating PDLSC towards a retinal fate [64]. The notion of PDLSCs as a good source for iPSC generation was supported by the cellular expression of c-Myc and OCT4 [65].

A typical cementum/PDL-like complex characterized by a layer of aligned cementum-like tissues and clearly associated PDL-like tissues can be generated after the transplantation of *ex vivo*-expanded PDLSCs into immunocompromised mice. The cementum/PDL-like structures have a completely distinct appearance compared with that of the typical dentin-pulp-like structures generated by DPSCs [22]. After transplantation into surgically created defects at the periodontal area of the mandibular molars in immunocompromised rats, a PDL-like tissue was regenerated, and PDLSCs were found to be closely associated with the alveolar bone, implying a potential functional role in periodontal tissue regeneration [22]. With HA/TCP as a carrier in the minipig model, transplanted PDLSCs generated a root/periodontal complex capable of supporting a porcelain crown, resulting in normal tooth function [25]. In addition, PDLSCs can be successfully implanted into a mesial dehiscence model in athymic rats where they promote periodontal tissue regeneration [66].

24.2.4 Dental Follicle Progenitor Cells

The dental follicle is an ectomesenchymal tissue that surrounds the developing tooth germ prior to eruption, which are commonly extracted and disposed of as medical waste; there are no ethical issues regarding DFPCs isolation. This tissue is thought to contain stem cells and lineage-committed progenitor cells for cementoblasts, periodontal ligament cells, and osteoblasts [23].

DFPCs have an extensive proliferative ability and are capable of forming hard tissue both *in vitro* and *in vivo* [23, 49]. Moreover, they express neural progenitor cell markers such as Notch-1 and nestin, and can form the tissues of the periodontium, including alveolar bone, PDL, and cementum [23].

Cultured DFPCs were demonstrated to exhibit osteogenic differentiation capacity under the appropriate conditions. Long-term cultures of DFPCs with dexamethasone produced compact calcified nodules or appeared as membrane-like structures [23]. Cementoblast features were detected in cultured DFPCs stimulated by BMP-2/-7 and enamel matrix derivatives [67]. Moreover, DFPCs could differentiate into chondrocytes and adipocytes, as illustrated by specific staining and the expression of specific markers [67]. The neural differentiation potential of DFPCs under *in vitro* conditions was therefore investigated [68]. Recently, DFPCs were reported to transdifferentiate into functional HLCs and acquire hepatocyte functions upon hepatogenic induction [37].

DFPCs combined with porous ceramic discs and transplanted into immunocompromised rats produced a cement/woven bone-like tissue with embedded cementocyte/osteocyte-like cells. However, no hard tissues formation, such as dentin, cementum, or bone, has been observed in the *in vivo* transplant [69]. Further studies are necessary to explore the potential for hard tissue regeneration.

24.2.5 Alveolar Bone-Derived Mesenchymal Stem Cells

Alveolar bone comprises the thickened ridge containing the tooth sockets in the bones that hold teeth, and it is embryonically derived from dental follicle. Recently, the successful isolation and culture of ABMSCs was manifested [24]. The isolated cells exhibit a spindle-shaped fibroblast-like morphology, plastic adherence, and colony formation. These cells express the surface markers as other dental stem cells do [24, 70, 71].

Expanded ABMSCs can differentiate into osteoblastic lineages, and they demonstrate high ALP expression [24]. Moreover, many studies have revealed that treatment of ABMSCs with the dichloromethane fraction of Dipsaci Radix [72], interferon-induced transmembrane protein 1 [73], nicotine [74], low-frequency pulsed electromagnetic fields [75], low-intensity pulsed ultrasound [76], low fluid dynamic shear stress [77], and orbital shear stress [78] could enhance osteogenesis in these cells. CS/HAp composite fabric may provide a good scaffold for ABMSC attachment, proliferation, migration, and differentiation for utilize in bone tissue engineering [79]. Additionally, ABMSCs showed chondrogenic and adipogenic differentiation potentials similar to those of other stem cell populations [71, 80].

ABMSCs induced significant new bone formation following subcutaneous transplantation into immune-deficient mice, and cuboidal osteoblasts and osteocytes appeared lining the surface along the margin of newly formed bone [24, 70, 71]. These data support the feasibility of using ABMSCs as a source of stem cells to treat bone tissue defects.

24.2.6 Stem Cells from the Apical Papilla

The apical papilla is the soft tissue found at the apices of developing permanent teeth [25, 81]. In developing teeth, root formation begins with the apical proliferation of epithelial cells from the cervical loop. The dental papilla contributes to tooth formation and is eventually converted into pulp tissue, and an apical cell-rich zone lies between the apical papilla and the pulp [81].

A unique population of MSCs referred to as SCAP was discovered in the apical papilla of human immature permanent teeth [25, 81]. SCAP show a higher proliferation rate and mineralization potential than DPSCs, and they express typical MSC markers, including STRO-1, CD73, CD90, CD105, and CD146 [25, 82] but they also express CD24, which could be used as a marker for this stem cell population. As neural crest associated cells, SCAP express a variety of neural cell markers like SHED [81]. Similar to DFPCs, SCAP represent a population of cells from a developing tissue and might thus exhibit greater plasticity than other DSCs.

Cultured SCAP can undergo adipogenic and odontogenic/osteoblastic differentiation following induction *in vitro*, analogous to the patterns exhibited by DPSCs and SHED [25]. Like DPSC and SHED, SCAP can be reprogrammed into iPSC [39]. Interestingly, *ex vivo*-expanded SCAP show positive staining for several neural markers without neurogenic stimulation [83]. After stimulation, additional neuron cell markers are also expressed by SCAP, including neuronal nuclear antigen, neurofilament M, and neuron-specific enolase [81]. In addition, SCAP demonstrated the capacity to differentiate into hepatocyte-like cells *in vitro* [37].

When *ex vivo*-expanded SCAP were transplanted into immunocompromised mice with an appropriate carrier matrix, a typical dentin-pulp-like complex was regenerated [25]. SCAP appear to be the source of the primary odontoblasts responsible for the formation of root dentin. In minipigs, transplanted SCAP and PDLSCs generated a bio-root periodontal complex capable of supporting a porcelain crown, resulting in functional tooth regeneration [25]. Human SCAP-mediated tissue regeneration may offer a promising cell-based therapy for root regeneration. Furthermore, SCAP can generate cement/woven bone-like tissue with embedded cementocyte/osteocyte-like cells *in vivo*. However, whether the material was dentin, cementum, or bone could not be identified [69].

24.2.7 Tooth Germ Progenitor Cells

TGPCs are a novel stem cell population identified in the dental mesenchyme of the third molar tooth germ during the late bell stage [26]. TGPCs can be expanded and maintained for nearly 60 population doublings, during which they retain their spindle-shaped morphology and high proliferation rate. TGPCs express the STRO-1 and CDs and demonstrate a tendency for pluripotency-associated gene expression (Nanog, Oct4, Sox2, Klf4, C-myc), indicating a mesenchymal phenotype [26, 84, 85].

TGPCs show a similar multilineage differentiation capacity to that of other dental MSCs, including the differentiation into adipocytes, osteoblasts/odontoblasts, chondrocytes, and neurons [26, 84–87]. Hepatic-induced TGPCs showed polygonal and epithelial-like morphology and strongly positive for liver-specific albumin gene. In addition, the immature hepatocyte marker AFP and the specific biliary epithelial cell marker CK19 were expressed more strongly during the culture period. These results indicated that TGPCs can differentiate into cells with the morphological, phenotypic, and functional characteristics of hepatocytes *in vitro* [26]. TGPCs form tube-like structures when incubated on Matrigel, which might indicate a possible contribution to vascularization [84].

TGPCs or TGPCs transfected with Venus were subcutaneously implanted with HA into immunocompromised rats. The HA/TGPC implants exhibited new bone formation in the presence of osteocytes in the newly formed bone matrix and a cuboid-shaped active osteoblast lining on the matrix surface. The implants with Venus-positive TGPCs were located within the mineralized matrix, where osteoblasts and osteocytes are typically found [26]. Cultured TGPCs show engraftment when they are transplanted *via* the portal vein into the liver of carbon tetrachloride (CCl_4)-treated rats. The transplantation of hepatic induction-treated TGPCs was effective in suppressing liver inflammation and fibrosis and reduced both the increase in bilirubin and the suppression of albumin. These findings suggest that the multipotent TGPCs are a candidate for cell-based therapy to treat liver diseases [26].

24.2.8 Gingiva-Derived Mesenchymal Stem Cells

The gingiva is a unique oral tissue overlaying the alveolar ridges and retromolar region that is recognized as a biological mucosal barrier and a distinct component of the oral mucosal immunity. And this tissue can often be obtained as a discarded biological sample [88]. Recently, GMSCs, a new population of stem cells isolated from human gingiva, were shown to exhibit clonogenicity, self-renewal, and multipotent differentiation capacity, and these cells possess both stem cell-like and immunomodulatory properties [27]. GMSCs fulfilled the criteria proposed by the ISCT for MSCs, they express CDs and display positive signals for Oct4, Sox2, Nanog, Nestin, SSEA4, and Stro-1 [27, 89, 90].

Some studies have claimed that GMSCs have multipotent MSCs properties after differentiating into multiple mesenchymal-derived cell types, such as adipocytes, chondrocytes, and osteoblasts, as determined by the increased expression of specific markers [27, 89–91]. The capacity of GMSCs to differentiate into a putative definitive endoderm (DE) lineage was further confirmed through demonstration of the expression of the DE markers Sox17, Foxα2, and CRCX4. When cultured on fibronectin-coated slides in endothelial cell growth medium, GMSCs expressed the endothelial cell marker CD31 [27]. Under neural differentiation medium, GMSCs are positive for GFAP, neurofilament 160/200 (NF-M), MAP2, nestin, and βIII-tubulin [27]. When subjected to a glial differentiation regimen, GMSCs induce neuritogenesis and support survival of PC12 cells in serum-free medium [92].

When transplanted with HA/TCP or fibrin gel as a carrier, GMSCs consistently regenerated connective tissue-like transplants that exhibited the histological features of the collagenous connective tissue phenotype, including the presence of fibroblast-like cells and collagen fibers. GMSCs have a potent *in vivo* self-renewal ability, as confirmed by serial transplantation in the same model [27, 89]. *Ex vivo*-expanded GMSCs were

seeded onto HA/TCP grafts, incubated in osteogenic medium, mixed with collagen gel, and transplanted subcutaneously into the dorsal surface of immunocompromised mice. High expression levels of osteocalcin, OPN and Col I were observed, indicating the potential of GMSCs for *in vivo* bone regeneration [27]. Newly formed bone with a well-mineralized trabecular structure was also demonstrated for GMSCs transplanted into the mandible and calvarial defect model [27, 90]. Surprisingly, dexamethasone-treated GMSCs implanted subcutaneously into SCID mice revealed the formation of bilineage (mesodermal and ectodermal) mixed tumors that included fetal fat, striated muscle, cartilage, bone, epithelial tissue, and neural tissue. This finding implies that GMSCs are capable of giving rise to tissues *in vivo* that develop from cranial neural crest cells during embryogenesis [92].

24.3 Potential Clinical Applications

Stem cell-based therapy for regenerative treatment is considered a promising treatment modality for future therapy. Dental tissues-derived stem cells are currently an excellent candidate for the regeneration of teeth and other organs, and treatment of inflammatory diseases, their progress in pre-clinical studies, and possible applications are outlined here [28].

24.3.1 Bone Regeneration

Dental tissue-derived MSCs have been used to engineer bone for orofacial bone regeneration.

DPSCs can produce a living autologous fibrous bone (LAB) tissue *in vitro*, which forms a lamellar bone with osteocytes after transplantation into immunocompromised rats [32]. DPSCs produced bone-like structures rather than dentin following transplantation *in vivo* with various carriers [45, 93]. A clinical application of DPSCs for human bone defects has been reported. DPSCs obtained from the mandibular third molars demonstrated the capacity to completely restore human mandible bone defects when they were transplanted with a collagen sponge scaffold [94].

SHED can repair critical-sized calvarial defects with robust bone formation when implanted *in vivo* [55]. As these cells are derived from neural crest cells, SHED may share a similar tissue origin with mandibular bone cells and therefore might be a suitable resource for the regeneration of alveolar and orofacial bone defects.

Although it is unclear whether transplanted cells directly differentiate into osteoblasts to create new bone, DFPCs clearly support new bone formation after transplantation into a surgically created, full-thickness, critically sized partial defect in immunodeficient rats [95].

Subcutaneous implantation of SCAP combined with HA scaffolds into immunocompromised rats formed bone-like mineralized tissues [93]. Furthermore, when SCAP were seeded onto synthetic scaffolds and then transplanted into immunodeficient mice, a continuous layer of dentin-like tissue was deposited onto the canal dentinal wall [96]. These findings provide evidence that SCAP can be used as an approachable stem cell source for bone formation.

GMSCs can repair mandibular wounds and calvarial defects *in vivo* with local implantation, implying that GMSCs could be a novel source of stem cell-based therapy during bone reconstruction [90].

24.3.2 Tooth Root Regeneration

Several clinical cases have demonstrated the role of apical papilla in root formation. A bio-root periodontal complex constructed with SCAP and PDLSCs was able to support an artificial porcelain crown to provide normal tooth function in a swine model, suggesting the feasibility of using a combination of autologous

SCAP/PDLSCs in conjunction with artificial dental crowns for functional tooth regeneration [25]. After root canal treatment, root-tip formation continued and the surviving SCAP appeared to produce odontoblasts responsible for complete root formation in several cases of apexogenesis in infected immature teeth with periradicular periodontitis or abscess, supporting a pivotal role for the apical papilla in root formation [97].

In addition, treated dentin matrix (TDM) was used as a natural biological scaffold for tooth root reconstruction in an animal model. TDM was able to induce and support DFPCs to develop root-like tissues with dentin-pulp-like tissues and cementum-periodontal complexes, implying successful tooth root regeneration [98]. Therefore, DFPCs could be utilized for the treatment of root or tooth defect or loss in the future.

24.3.3 Dentin-Pulp Regeneration

The use of DPSCs, SHED, and SCAP for dentin-pulp tissue regeneration has been investigated.

A study using human tooth root fragments with an empty root canal space indicated the regeneration of vascularized dentin- pulp-like tissue when the canal was filled with PLG scaffold seeded with DPSCs [96]. When preameloblast culture medium treated DPSCs were transplanted into immunocompromised mice, they generated pulp-like structures lined with odontoblast-like cells [99]. These studies have primarily demonstrated the potential of complete pulp regeneration under experimental conditions.

A pulp-like tissue with well-established vascularity was induced and a continuous layer of dentin-like tissue was deposited onto the canal dentinal wall when SCAP were inserted into tooth fragments and then transplanted into immunodeficient mice [96, 97]. These data provide evidence that SCAP can be used for tissue engineering and regeneration.

Xenogeneic transplants containing SHED with HA/TCP generated donor-derived dentin-pulp-like tissues with distinct odontoblast layers lining the mineralized dentin-matrix [60]. SHED seeded onto biodegradable scaffolds prepared within human tooth slices successfully differentiated into odontoblast-like cells with a cytoplasmic process extending into a dentinal tubule, suggesting that SHED constitute a viable source of cells for dental pulp tissue engineering [53]. SHED seeded onto polylactic acid scaffolds with the addition of BMP-2 and TGF-β1 produced pulp tissue constructs, suggesting that future regenerative endodontic treatment may involve the cleaning and shaping of root canals followed by the implantation of vital dental pulp tissue constructs created in the laboratory [100].

The establishment of reproducible and safe methods for regenerating dentin-pulp complexes to achieve the desired pulp tissue regeneration is expected.

24.3.4 Periodontal Regeneration

Stem cell-based regenerative periodontal therapy has gained attention since the isolation of MSCs from various tissues.

The bone morphogenetic protein BMP-2 has been used to promote the differentiation of DFPCs into cementoblasts and odontoblasts to reestablish the integrity of the PDL [101].

Due to their periodontal ligament derivation and the capacity to differentiate into osteoblasts, cementoblasts, and fibroblasts, PDLSCs may be the first candidate cellular source for PDL regeneration. It has been demonstrated that expanded PDLSCs are capable of regenerating a typical cementum/periodontal ligament-like structure with HA/TCP as a carrier [22]. Extensive research exploring the potential use of PDLSCs to treat periodontal diseases in various larger animal models is ongoing. Several pilot studies have demonstrated that transplantation of PDL cell sheets was able to regenerate periodontal tissue in experimental defect models [102]. In addition to animal models, a retrospective pilot study in humans has also demonstrated the therapeutic benefit of autologous periodontal ligament progenitor cells (PDLPs) when implanted with bone grafting material into intrabony defects in patients. All periodontal defects were reconstructed, and the

experimental and clinical evidences support the potential efficacy and safety of utilizing autologous PDL cells in the treatment of human periodontitis [103]. Furthermore, one human clinical study has demonstrated the development of new tissue consistent with PDL on the surface of dental implants. This proof-of-principal investigation shows great potential and may provide efficient methods for enhancing the outcome of implant treatment using PDLSCs [104].

An optimal protocol for the extraction, expansion, and characterization of human PDL cells has been demonstrated, and the safety and efficacy of the PDL sheet for clinical trials has been validated [105]. One clinical trial using PDL sheet technology is currently under way to develop a government-approved periodontal cell transplantation therapy [106].

24.3.5 Neurological Disease

Transplanted DPSCs can survive [35] and may induce neuroplasticity [107] in the central nervous system. Injection of DPSCs into the right dorsolateral striatum of experimental animals subjected to middle cerebral artery occlusion (MCAO) induced a significant recovery from neurological dysfunction [108]. SHED could be induced to form neural-like spheres in a medium optimized for neural stem cells *in vitro*. Additionally, transplantation of SHED spheres into parkinsonian rats partially improved the apomorphine-evoked rotation of behavioral disorders, suggesting that SHED may be a promising source for the treatment of neurodegenerative diseases [109]. In a recent study, neuronally pre-differentiated DPSCs were injected into the cerebrospinal fluid of rats with induced cortical lesions, these cells integrated into the host brain and exhibited neuronal properties, indicating that they may serve as useful sources of neuro- and gliogenesis *in vivo* [110].

Recently, three independent groups reported that pulp stem cells showed neuroregenerative activity in rodent spinal cord injury (SCI) models. Human dental pulp cells (HDPCs) transplanted into a mouse model of compressive spinal cord injury showed higher levels of trophic-factor expression in the tissue, better tissue organization, and the presence of many axons or oligodendrocytes and neurons with synapses, indicating that HDPCs may be feasible candidates for therapeutic intervention after SCI and during central nervous system disorders in humans [111]. Functional recovery was promoted when undifferentiated or neural-induced SHED were transplanted into a rat spinal cord contusion injury model, suggesting that engrafted SHED or their derivatives could be suitable candidates for the treatment of SCI and other neurodegenerative diseases [112]. Transplantation of SHED into the completely transected adult rat spinal cord significantly improved the recovery of hind limb locomotor functions. Thus, engrafted SHED may provide therapeutic benefits for treating SCI through both cell-autonomous and paracrine neuroregenerative activities [113].

A recent study indicated that TGPCs utilize angiogenic anti-oxidative and anti-apoptotic mechanisms to exert neuroprotective effects on *in vitro* models of Alzheimer's dementia (AD) and Parkinson's disease (PD), which might provide insight into the therapeutic potential of TGPCs as a cellular treatment for neurological disorders [114]. Injection of PDLSCs into a rat model of crushed nerve injury improved axonal regeneration and recovered sensory function, comparable with Schwann cell therapy [115].

These reports suggest that dental stem cells offer valuable therapeutic potential in the central nervous system.

24.3.6 Lesions of the Cornea

DPSCs produced corneal stromal extracellular matrix containing type I collagen and keratocan after injection *in vivo* into mouse corneal stroma, and did not affect corneal transparency or induce immunological rejection, demonstrating a potential for the clinical application of DPSCs in cellular or tissue engineering therapies for corneal stromal blindness [40]. SHED are capable of reconstructing the eye surface following the induction of unilateral total limbal stem cell deficiency in rabbits, suggesting that SHED might be used as a potential alternative source of cells for corneal reconstruction [116]. Transplantation of a tissue-engineered cell sheet

composed of human SHED into rabbits with experimentally induced corneal defects resulted in successful reconstruction of the corneal epithelium [117].

24.3.7 Regeneration of Other Non-Dental Tissues

Subcutaneous transplantation of human PDLSCs can lead to the formation of substantial amounts of collagen fibers and improve facial wrinkles in mice [118].

The induction of smooth and skeletal muscle cells from human SHED *in vivo* has been reported [46, 52]. Systemic application of SHED to animals suffering from muscular dystrophy improves the clinical symptoms [46].

DPSCs can repair infarcted myocardium associated with an increase in the number of vessels and a reduction in the infarct size, probably due to their ability to secrete proangiogenic and anti-apoptotic factors. Therefore, this study suggests that DPSCs could provide a novel alternative cell population for the treatment of ischemic diseases [119].

When TGPCs were transplanted into CCl_4-treated liver-injured rats, they prevented the progression of liver fibrosis and contributed to the restoration of liver function. These findings suggest that TGPCs are a candidate for cell-based therapy to cure liver diseases and offer unprecedented opportunities for developing therapies to facilitate tissue repair and regeneration [26].

VEGF-induced DPSCs maintain endothelial cell-like features when cultured in a 3-D fibrin mesh, displaying focal organization into capillary-like structures [120]. DPSCs were reported to show an increase in blood flow with a high density of capillary formation during experimentally induced mouse ischemia, suggesting their potential for promoting angiogenesis/vasculogenesis [121].

Transplantation of DPSCs ameliorated ischemic tissue injury in the rat brain and accelerated functional recovery after MCAO, indicating that DPSCs could be a potential candidate for the treatment of stroke [122]. SHED-conditioned medium promoted the migration and differentiation of endogenous NPCs, induced vasculogenesis, and ameliorated ischemic brain injury after intranasal administration to SD rats subjected to permanent MCAO [123].

24.3.8 Inflammatory and Allergic Diseases

Systemic delivery of SHED resulted in significant engraftment in the muscles of dogs with golden retriever muscular dystrophy (GRMD), which is likely due to the immunomodulatory effect of SHED. Both the increase of cell engraftment with consecutive SHED transplantation and the absence of an immunological response in the GRMD dog model indicate important implications in designing future therapeutic trials [46]. In addition, systemic infusion of SHED was able to effectively reverse systemic lupus erythematosus (SLE)-associated disorders, possibly because of their superior immunomodulatory effects that promote the recovery of the ratio between Tregs and Th17 cells. These data suggest that SHED may be an accessible and feasible source of MSCs for treating immune disorders, such as SLE [124].

Cell-based therapy using a systemic infusion of GMSCs significantly ameliorated the severity of inflammation-related colonic injuries in experimental colitis by suppressing inflammatory cell infiltration and inflammatory cytokine/mediator secretion and increasing Treg accumulation and IL-10 expression at local intestinal sites [27]. In a murine excisional full-thickness skin wound model, systemic infusion of GMSCs significantly enhanced the repair process, as indicated by more rapid re-epithelialization and increased angiogenesis, providing evidence that GMSCs are a promising cell source for stem cell-based therapies of inflammatory diseases and skin wounds [89]. Systemic infusion of GMSCs also dramatically suppressed CHS via PGE(2)-dependent mechanisms before the sensitization and challenge phase [125]. In addition, systemic infusion of GMSCs mitigated chemotherapy-induced oral mucositis in murine models, as demonstrated by the reversal of body weight loss and the stimulation of regeneration in the disrupted epithelial lining [126]. Infusion of GMSCs into mice

with CIA significantly reduced arthritis severity, decreased histopathology scores, and down-regulated the production of inflammatory cytokines (IFN-γ and IL-17A), suggesting that GMSCs provide a promising approach for the treatment of autoimmune diseases [127]. Taken together, GMSCs can function as an immunomodulatory and anti-inflammatory component of the immune system *in vivo* and represent a promising and easily accessible cell source for MSC-based therapies to treat inflammatory and allergic diseases.

24.4 Safety

The populations of dental stem cells are extremely attractive cell sources for lineage cell differentiation and tissue regeneration. However, the risk of immunogenicity and rejection, tumor formation and ethical considerations have restricted their use to mainly in vitro experimental studies and their therapeutic potential remains to be determined. It is needed to evaluate the risks of dental stem cells isolation, manipulation, transplantation, immunogenicity and rejection, and tumor formation for the future usage of regenerative medicine.

24.4.1 Immune Rejection

Immunocompatibility is the most important concern for the clinical application of dental stem cells. The normal functions of the recipient immune system can be interfered if there exit a rejection of donor DSCs. However, various preclinical studies that used DSCs already claimed the absence or a very low immunogenic response of the recipient organism, and the immunomodulatory properties similar to MSCs [46, 111, 117, 128].

Previous reports have demonstrated that DPSCs can suppress T-cell proliferation and therefore might be suitable for preventing or treating T-cell alloreactivity associated with hematopoietic or solid-organ allogeneic transplantation [129]. *Ex vivo*-expanded DPSCs significantly inhibited the proliferation of peripheral blood mononuclear cells (PBMCs) via the expression of soluble factors partly induced by the secretion of interferon (IFN)-γ by activated PBMCs [59]. In another study, Toll-like receptors (TLRs), key molecules that bridge the innate and adaptive immune responses, were shown to trigger the immunosuppression of DPSCs by up-regulating the expression of transforming growth factor (TGF)-β and interleukin (IL)-6 [130]. In addition, DPSCs could induce activated T-cell apoptosis *in vitro* and ameliorate inflammation-related tissue injuries in mice with colitis, which was associated with the expression of the Fas ligand (FasL). Knockdown of FasL expression reduced the immunoregulatory properties of DPSCs in the context of inducing T-cell apoptosis [131].

SHED significantly inhibited T helper 17 (Th17) cell differentiation but increased the number of regulatory T-cells (Tregs) *in vitro*.

Previous studies have shown that activated human PBMCs induced PDLSCs to secrete soluble factors, including TGF-β, HGF and IDO, that partly suppress PBMC proliferation [59]. PDLSCs were discovered to possess low immunogenicity and marked immunosuppressive activity via prostaglandin E2 (PGE2)-induced T-cell energy [102]. Furthermore, a recent study reported that PDLSCs isolated from inflamed periodontium showed significantly diminished inhibitory effects on the proliferation index of T-cells compared to those of healthy cells. In cocultures, stimulated PBMCs showed a significant decrease in the induction of Tregs, suppression of Th17 differentiation, and secretion of IL-10 and IL-17 in the presence of inflamed PDLSCs compared with healthy PDLSCs, demonstrating that inflamed PDLSCs had markedly dysfunctional immunomodulatory properties, which may explain the pathogenesis of periodontitis and facilitate the development of therapies for this condition [132].

Recent studies have shown that DFPCs produced TGF-β and suppressed the proliferation of PBMCs. Treatment with TLR3 and TLR4 agonists augmented the suppressive potential of DFPCs and potentiated TGF-β and IL-6 secretions [130, 133]. These properties of DFPCs are desirable for the treatment of diseases caused by chronic inflammation accompanied by tissue injury [130].

SCAP possess low immunogenicity and can inhibit T-cell proliferation *in vitro* through an apoptosis-independent mechanism. SCAP can also suppress the one-way mixed lymphocyte reaction (MLR) in a dose-dependent manner. Certain soluble factors may be involved in SCAP-mediated immune suppression, but the exact mechanisms require further study [134]. In addition, cryopreservation did not affect the immune properties of SCAP [82].

GMSCs are capable of eliciting a potent inhibitory effect on T-cell proliferation in response to mitogen stimulation. Mechanistically, GMSCs exert their anti-inflammatory effect partly via the IFN-γ-induced expression of IDO, IL-10, cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS), which are known immunosuppressive factors. In addition, GMSCs can elicit M2 polarization of macrophages characterized by an increased expression of the mannose receptor (MR; CD206) and the secretory cytokines IL-10 and IL-6 and decreased induction of Th 17 cell expansion, which might contribute to a marked acceleration of wound healing [135].

Even if dental stem cells seemingly are not rejected by the immune system, the understanding of their immunomodulatory properties has great relevance for DSCs proper clinical use.

24.4.2 Tumor Formation

Tumor formation after stem cell transplantation mainly depends on the cell type, its proliferative and migration capacity, as well as on the site of injection. It has been shown that adipose-derived stem cells (ASCs) contributing to neovasculature formation *in vivo*, which feeds the growth of tumors [136]. Interestingly, DSCs when appropriately cultivated rarely suffer malignant transformation, even after long-term *in vitro* culture. Several animal experiments have shown that DSCs do not form tumors after transplantation [137]. This maybe because of DPSCs are isolated from a perivascular niche in the dental pulp, which minimizing the risk of tumor formation [138]. Even if, we should take full consideration of the difficulty to assess the risks in preclinical studies on tumor formation after transplantation once such events occur after a long time.

24.5 Dental Stem Cell Banking

Dental stem cells appear to be a promising source for the treatment of various ailments already discussed herein. Personalized medicine is strongly believed to be the most promising avenue for treating challenging diseases and injuries throughout life as it provides a guaranteed matching donor; however, the use of an individual's own dental stem cells during a time of therapeutic necessity has serious limitations because it requires the extraction of remaining teeth. Thus, the ability to harvest and safely store stem cells from deciduous teeth and extracted permanent teeth is important to provide the greatest future benefit for life. Individuals have various opportunities at each stage of their life to bank these valuable cells, and it is best to recover stem cells from young and healthy individuals when the cells are strong and proliferative. Once stem-cell-containing tissues, such as pulp, apical papilla, periodontal ligament, follicle, gingiva, or the tooth itself, have been obtained from the patient, they can be cryopreserved for many years to retain their regenerative potential for use in future regenerative therapies [28].

Tooth banking is not currently a popular practice, but the trend is catching up mainly in developed countries. Current licensed tooth banks include the following [139]:

Advanced Center for Tissue Engineering, Japan (www.acte-group.com/)
Teeth Bank Co., Japan (www.teethbank.jp/)
BioEDEN, USA (www.bioeden.com/)
StemSave, USA (www.stemsave.com/)

Store-A-Tooth, USA (www.store-a-tooth.com/)
Stemade Biotech Pvt., India (www.stemade.com/)
The Norwegian Tooth Bank, Norway (www.fhi.no/morogbarn)

Although the autologous transplantation of banked teeth has been successfully achieved in the clinic, stem cell-based therapies involving stem cell banking have not yet been reported, and still human research trials with banked stem cells are needed to document the same results in humans. Moreover, many ethical controversies and legal and social questions need to be addressed before banked dental stem cells become clinically available. Therefore, the utility of stem cells isolation, purify, growth, and banking should be carefully evaluated and proper maintenance of the cryopreserved cells and tissues to ensure good quality for future use in transplantation should be ensured. In addition, legislation of the banking system is essential, as it may provide bio-insurance for future use [28].

24.6 Conclusions and Perspective

Current research on dental stem cells is expanding at an unprecedented rate. Stem cells derived from teeth are easily accessible and can be obtained in a convenient and minimally invasive way. According to this discussion, these new sources of stem cells could be beneficial for cellular therapy and the eventual development of regenerative treatment. These cells guarantee a donor match (autologous transplant) for life that, to a certain extent, may also be useful for close relatives of the donor. In addition, because these are adult stem cells and are not the subject of the same ethical concerns as embryonic stem cells [28].

However, several main objectives need to be addressed for future research, especially concerning the following issues [28]:

Identifying specific surface markers and understanding the mechanisms of self-renewal. This understanding would allow us to purify, regulate, and expand dental stem cells growth in the lab to generate sufficient numbers of cells for therapeutic use [28].

Understanding the regulation of DSCs during differentiation and the generation of specific tissue. The formation of certain tissues requires the expression of particular genes and involves sequential signal activation. Controlling these signals in the correct temporal pattern may facilitate regeneration of the desired tissue both *in vitro* and *in vivo* [28].

Clarifying the long-term fate of transplanted DSCs in the recipient. The capacity and efficiency of DSCs for homing to and transdifferentiating into a particular tissue and their ability to find the optimum "niche" have been major concerns [28].

Understanding how the various functional attributes of MSCs are specified at the population level. MSC populations display considerable phenotypic and functional heterogeneity [140]. BMMSCs are the most extensively investigated population and these cells are heterogeneous [141]. We assume that intrinsic heterogeneity may also exist in DSCs. Multiple methods can be used to understand the molecular basis of heterogeneity and the impact of heterogeneity on the clinical development and therapeutic potency of MSCs. This understanding will allow future studies to enhance the clinical efficacy of stem cell-based therapies [28].

Clarifying the mechanisms underlying the immunomodulatory properties of DSCs. DSCs offer a fascinating new cell source for clinical applications; however, the immune responses of the recipient should be noted. Further research will explore the critical role of DSCs in immunomodulation and the secretion of DSCs, as well as the interactions between DSCs and the immune system for future applications [28].

Identifying whether DSCs are subject to the effects of aging due to intrinsic factors and the somatic environment or not. Age-related changes can impinge on the activity of MSCs, as shown in previous studies [142]. Hence, discovering biomarkers to assess the cellular senescent state, establishing reliable methods to

measure age-induced effects, and identifying the molecular basis of dental stem cell aging will undoubtedly affect their future clinical use [28].

Understanding the relationships between MSCs senescence and organismal aging and developing approaches to reverse the effects of aging on MSCs are recommended for future studies [28].

Developing safe and reproducible delivery systems for depositing DSCs into the recipient and designing a comprehensive and global strategy for dealing with a multiplicity of issues involving the safety, harmonization, privacy, and transparency. The emerging trend of establishing human dental stem cell banks to support basic research and translational applications requires international collaboration. It will be important to address harmonization and standardization processes for banking dental stem cells and establish an international banking network based on common fundamental norms and standards, which will undoubtedly ensure the legitimacy of these banks [28].

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Stem Cells in the Skin

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25.1 Introduction

25.1.1 Skin Structure

Skin is the largest organ in the human body, representing approximately one-tenth of the body mass. It features a range of complex structures composed of the epidermis (epithelial tissue), dermis (connective tissues), and an underlying hypodermis that is not technically part of the skin. The epidermis is the outermost covering; the majority of cells in the skin epidermis are keratinocytes, although it also contains melanocytes, Langerhans cells, and Merkel cells.

The epidermis is composed of several layers of keratinocytes – basal, spinous, granular, and cornified layers, from bottom to top, which are at various stages of terminal differentiation. The topmost layer is dead cells and sheds continuously, and this layer is progressively replaced by new generated keratinocytes that divide from the basal layer. The dermis is situated below the epidermis and consists of connective tissue populated with fibroblasts. The dermis provides cushioning and tensile strength to the skin through an extracellular matrix consisting of collagen fiber bundles in a basket weave arrangement, all embedded within proteoglycans.

25.1.2 Skin Physiological Functions

The skin has three main functions: protection, regulation, and sensation. Protection is the major function of human skin. Skin acts as a barrier, it can protect against various environment stimuli, including mechanical impact and pressure, variations in temperature, microorganisms, radiation, and chemicals. Regulation is

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another function of human skin; it regulates body temperature via sweat and hair, it maintains an internal balance of water and salt via sweat. It also acts as a reservoir for the synthesis of vitamin D. The skin is an organ of sensation. It contains an extensive network of nerve cells that are sensitive to changes in the environment; this function relies on the separated receptors for heat, cold, touch, and pain, which are widely distributed throughout the body [1].

25.1.3 Skin Regeneration and Skin Stem Cells

The physiological functions of human skin rely on its constant renewal throughout adult life. In adult mammalian organisms, regeneration is commonly seen in multiple tissues, including skin, blood, stomach, and intestines. The regeneration process involves the constant shedding of older cells and replacement by new differentiated cells, which are consistently regenerated from resident stem cells. Skin regeneration is the process of renewing the surface of the skin; this process relies on the activity of skin tissue specific stem cells. Stem cells (SCs) are a unique population of cells that possess the capabilities of self-renewal and differentiation into specialized cell types.

25.2 Stem Cells in the Skin

Stem cells are classified into several different types for this different differentiation capacity. Totipotent stem cells show excellent ability to differentiate into whole tissues and these stem cells normally refer to the fertilized eggs of humans or animals. Pluripotent stem cells are stem cells that have the ability to differentiate into ectoderm, mesoderm, and endoderm germ layers. These include embryonic stem cells and induced pluripotent stem cells [2]. Multipotent stem cells are able to differentiate into multiple types of structures with specialized functions in the body, such as hair follicle stem cells (HFSC) in the skin, which can differentiate into hair follicles, epidermis, sebaceous glands, and neurons. Unipotent stem cells that are able to differentiate into one cell type, such as epidermal stem cells, could regenerate a differentiated epidermis [2].

The epidermis consists of multiple different progenitor cell populations, all of which are important to epidermal function. In a normal epidermis, skin stem cells constitute approximately 1–10% of the basal stratum. It is believed that skin is the tissue that has the richest amount of stem cells of all human organs. Like other adult stem cells, epidermal stem cells can differentiate into specialized cells and can divide (through mitosis) to produce more stem cells. The stem cells in the epidermis undergo asymmetric divisions, two distinct daughter cells with different cellular fates will be generated, one copy of the original stem cell as well as a second daughter cell programmed to differentiate into a non-stem cell fate. The later ones are called transient amplifying cells (TACs), which undergo a limited number of mitotic divisions and ultimately differentiate.

Various populations of stem cells have been identified in different regions of the epidermis; they maintain homeostasis and the renewal of the entire skin epithelium, as well as play critical roles in skin repair after injuries. The mammalian epidermis consists of three self-renewing compartments: the hair follicle, the sebaceous gland, and the interfollicular epidermis. In supporting these biological functions, stem cells in the epidermis can be divided into several heterogeneous populations with different homing sites. One epidermis stem cell population is the Interfollicular Epidermal Stem Cells (IFESCs), located in the interfollicular parts of the epidermis. Another population of skin stem cells located in the region of the outer root sheath of the hair follicle (bulge), is called hair follicle stem cells (HFSCs). And there are other stem cell populations, including sebaceous stem cells, melanocyte stem cells, dermal resident mesenchymal stem cells, and adipose-derived stem cells.

25.2.1 Epidermal Stem Cells

25.2.1.1 Hair Follicle Stem Cells (HFSCs)

A promising source of adult stem cells is from hair follicles. Hair follicles are a niche of various stem cell populations and a major source of cells responsible for regeneration of hair, sebaceous glands, and epidermis. HFSCs and their progenitors are directly involved in hair and skin regeneration during skin homeostasis. Based on their anatomical classification and function, HFSCs are mainly referred to as epithelial stem cells and have been broadly known as epidermal stem cells, as well as bulge stem cells in diverse studies [3]. Hair follicle stem cells exist in the lower end bulb region of anagen hair follicles. These cells are highly active, which can give rise to hair fibers through rapid proliferation and complex differentiation [4]. HFSCs can give rise to keratinocytes, sebocytes, and transient amplifying progenitor cells. These cells were well characterized in the early 1990s, when it was found that label-retaining cells were located in the upper portion of the hair follicle (bulge area) [5]. In the following studies, it has been confirmed using transgenic label-retaining assays that some hair follicle cells were able to generate all epidermal lineages when transplanted [6]. Subsequently, it was clarified that during the early anagen phase hair follicle cells grew downward in response to the stimulating factors of the dermal papilla cells and formed half a hair follicle, which was degenerated during the catagen phase.

In a very recent mouse study, it was found that mouse HFSCs stay in a fixed location in the hair follicle called the bulge area; HFSCs continuously generate new cells to the hair bulb during the anagen phase. Due to their excellent regenerative ability to differentiate into most of the ectodermal lineages, HFSCs have been considered a promising cell source for grafting in various skin diseases.

25.2.1.2 The Interfollicular Epidermal Stem Cells

Interfollicular epidermal stem cells closely adhere to the basal lamina of the epidermis. They resemble somatic SCs. The slow cell cycle of these cells prevents the accumulation of mutations, a long life span, and proliferation ability providing maintenance and repair of the tissue in which they reside. The final differentiation into keratinocytes begins after loss of contact with the basement membrane and is related to renewal of the epidermis.

By using DNA labeling and cell turnover studies, it has been demonstrated that interfollicular epidermal stem cells are interspersed through the basal layer, each small unit of epithelial tissue comprises about 10 basal cells and their suprabasal maturing progeny are responsible for repopulating; this is termed the epidermal proliferative unit (EPU). The slow cycling interfollicular epidermal cell, initially characterized by its ability to retain 3H-Tdr incorporation in its DNA in the longer term, is located in the center of the EPUs. In comparison, the rapidly proliferating cells have been quantified as transit amplifying (TA) cells, located in the peripheral region of the EPU, and these cells are the differentiated daughter cells of interfollicular epidermal stem cells [7]. By Brdu staining, the existence of a single stem cell dotted throughout the murine interfollicular epidermis was recently confirmed [8]. This result indicates that the existence of interfollicular epidermal stem cells and their renewal function is the only cause of their consistent self-renewal.

25.2.1.3 Sebaceous Stem Cells

The sebaceous gland (SG) is an epidermal appendage that is important in skin barrier function and is involved in common skin diseases, such as acne vulgaris and androgenic alopecia. The sebaceous gland is usually closely associated with hair follicles, forming a pilosebaceous unit. The predominant cells in the sebaceous gland, sebocytes, secrete lipid-rich products into the infundibular opening of the adjacent hair follicle [9]. Recently, sebaceous gland stem cells have been identified, cells expressing the transcriptional repressor

Blimp1 have been identified as sebocyte progenitors [8]. These progenitor cells were considered to be specific sebaceous gland stem cells, since Blimp1+ progenitors give rise to terminally differentiated Pparγ+ sebocytes via transient amplifying progenitors and they do not contribute progeny towards interfollicular epidermis or hair follicles [9]. Blimp1 seems to play a rate-limiting role in regulating sebaceous gland homeostasis as epithelial deletion of Blimp1 leads to sebaceous gland hypertrophy and an oily hair coat phenotype [10].

25.2.1.4 Melanocyte Stem Cells

Melanocytes in the skin play an indispensable role in the pigmentation of skin and its appendages. Melanocytes produce pigment granules that color both skin and hair. It is generally believed that the embryonic origin of melanocytes is neural crest cells; however, in adult skin, functional melanocytes are continuously repopulated by the differentiation of melanocyte stem cells (McSCs) residing in the epidermis of the skin [11]. Melanocyte stem cells (McSCs) are stored in hair bulges or sub-bulge regions and function as a melanocyte reservoir. In normal hair follicles, HFSCs and McSCs are frequently found in these stem cell niches, Quiescence, maintenance, activation, and proliferation of McSCs are controlled by specific activities in the microenvironment that can influence the differentiation and regeneration of melanocytes [12]. During the telogen phase of the hair cycle, both HFSCs and McSCs remain quiescent. However, when a new hair cycle begins, these two stem cell populations are activated by increased Wnt signaling and decreased TGF-β signaling, which crosstalk with signaling molecules provided by dermal papilla at the base of a hair follicle, resulting in McSCs proliferation and differentiation [13, 14]. In response to wounding or UV irradiation, follicular McSCs can exit the stem cell niche before their initial cell division, migrate toward the basal layer of the epidermis in a melanocortin 1 receptor (Mc1r)-dependent manner, and differentiate into functional epidermal melanocytes [15]. In a clinical case, narrow-band ultraviolet B (UVB) exposure is commonly used for the treatment of patients with vitiligo; the principle is to stimulate the proliferation, migration, and differentiation of McSCs that lead to follicular repigmentation of depigmented skin [16].

25.2.2 Stem Cells in the Dermals

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are a multipotent cell type that arises from the embryonic connective tissue mesenchyme. Their multipotent properties enable them to readily differentiate into several different cell types including osteoblasts, chondrocytes, adipocytes, tenocytes, and myocytes under specific culture conditions. Bone marrow is the prominent source but MSCs have in fact been found in various niches throughout the body. Modern basic scientific research evidence has demonstrated the presence of MSCs in the dermis. This specific mesenchymal stem cell population existing in the human dermal layer was named dermal mesenchymal stem cells (DMSCs) or cutaneous mesenchymal stem cells (Cutaneous MSCs). The research published by Toma et al. was the first to isolate multipotent adult stem cells from the dermis [17]. The authors successfully isolated a "skin-derived precursor" cell population, since these cells cannot only generate mesodermal progeny (adipocytes and smooth muscle cells) *in vitro* but can also be differentiated into neurons *in vitro*; thus, these cells were essentially defined as stem cells.

Following these studies, there has been an increasing number of reports confirming the existence of mesenchymal stem cells in other species, such as porcine [18], murine [19], and also recently the human dermis [20]. In the skin, MSCs are predominantly found in the dermal papilla, functioning in secreting diverse growth factors after wounding to promote fibroblast proliferation and collagen formation and to elicit intrinsic stem cell differentiation [21], serving as a modulator to activate macrophages, and directly affecting hair follicle morphogenesis [22]. In human studies, it has been found that cutaneous mesenchymal stem cells have multi-lineage differentiation potential; these cells can differentiate into adipocytes and osteocytes, or additionally into a chondrogenic phenotype [21].

25.2.3 Stem Cells in the Subcutaneous Tissue

Within the adult stem cell population, adipose-derived stem cells (ADSCs) are one of the most promising stem cell types. ADSCs are found in any type of white adipose tissue, including subcutaneous and omental fat [23]. Skin adipose-derived stem cells normally refer to the subcutaneous adipose-derived stem cells. ADSCs are clinically attractive because they can be easily extracted in large amounts from liposuction aspirates or subcutaneous adipose tissue fragments and possess a high recovery yield. And there are no ethical concerns such as those of human ESCs for their use in diverse clinical applications [24]. ADSCs are multipotent and can differentiate into adipocytes, osteoblasts, chondrocytes, myocytes, and neuronal cells. Due to multipotency of the ADSCs, they can be used widely in various clinical applications.

25.3 Isolation and the Biological Markers of Skin Stem Cells

Stem cells are described as cells that have the ability to undergo self-renewal, be proliferative, and differentiate into multiple lineages. Over the past decade, researchers in the stem cell field have identified and developed ways to detect and isolate skin stem cells. The pioneering studies from hemopoietic stem cell research suggested that the adult stem cells are infrequently dividing (slowly cycling), quiescent cells, which therefore retain radioactively labeled nucleotides, such as titriated thymidine or Brdu [25], In skin, such cells are also named label-retaining cells (LRCs) [26].

In vivo animal models provide a useful tool to identify skin stem cells, the study of the cutaneous stem cells started by identification of quiescent "label retaining cells" in mice models. To isolate live LRCs using flow cytometry, Fuchs and colleagues elegantly adapted a pulse-chase technique to visualize LRCs by green fluorescent protein tagged histone (H2BGFP) [27]. In recent years, Genetic Lineage Tracing technology has been widely used in stem cell biology. Lineage tracing using the Cre-loxP system enables genetic labeling of stem cells and their progeny in an intact condition, undamaged tissue using fluorescent and other reporters. The most commonly used system for tracing mouse skin stem cells involves a transgenic mouse harboring Cre recombinase under the control of the epidermal basal layer-specific keratin 14 promoter (K14CreERt). This mouse line has been further crossed with a mouse ubiquitously expressing various reporters such as LacZ, or a fluorescent marker flanked by an loxP sequence, thus meaning that the location and features of adult epidermal stem cells can be directly observed.

The best characterized stem cell population in the epidermis, and likely the most pluripotent, is the quiescent stem cells in the hair follicle bulge (Fig. 25.1/Plate 29). In hair follicles, numerous cell surface markers have been identified to trace the subsets of stem cell populations with varying differentiation potentials. These included Epi-NCSCs, nestin and keratin 15, CD34, CD200, and the G protein-coupled receptor 5 (Lgr5) [28, 29]. Hair follicle stem cells can be routinely isolated from hair follicles and expanded *in vitro* to cell populations that are similar to the adult stem cells with respect to morphology and cell surface markers. HFSCs normally give rise to keratinocytes, sebocytes, and transient amplifying progenitor cells. In mouse hair follicle bulges, hair follicle stem cells seem to express specific markers including CD34 and K19, as well as CD200. Expression of CD200 has been found to protect the bulge area from inflammation and hair loss in alopecia areata in a mouse model, and human bulge stem cells also expressed similar cell surface markers [5]. CD34 seems to be the most reliable cell surface marker for human hair follicle stem cells and this cell surface marker has been successfully used to identify and isolate hair follicle stem cells by using fluorescence-activated cell sorting (FACS) and magnetic activated cell sorting (MACS). More recently, Snippert et al. [30] reported the expression of Lgr6 in HFSCs, which differs from Lgr5/CD34+ HFSCs. In adult hair follicles, Lgr6+ cells reside in a previously uncharacterized region directly above the follicle bulge. These cells do not express any known bulge stem cell markers. In contrast, Lgr6+ HFSCs particularly express Sca-1, α6-integrin, β1-integrin, sox9,

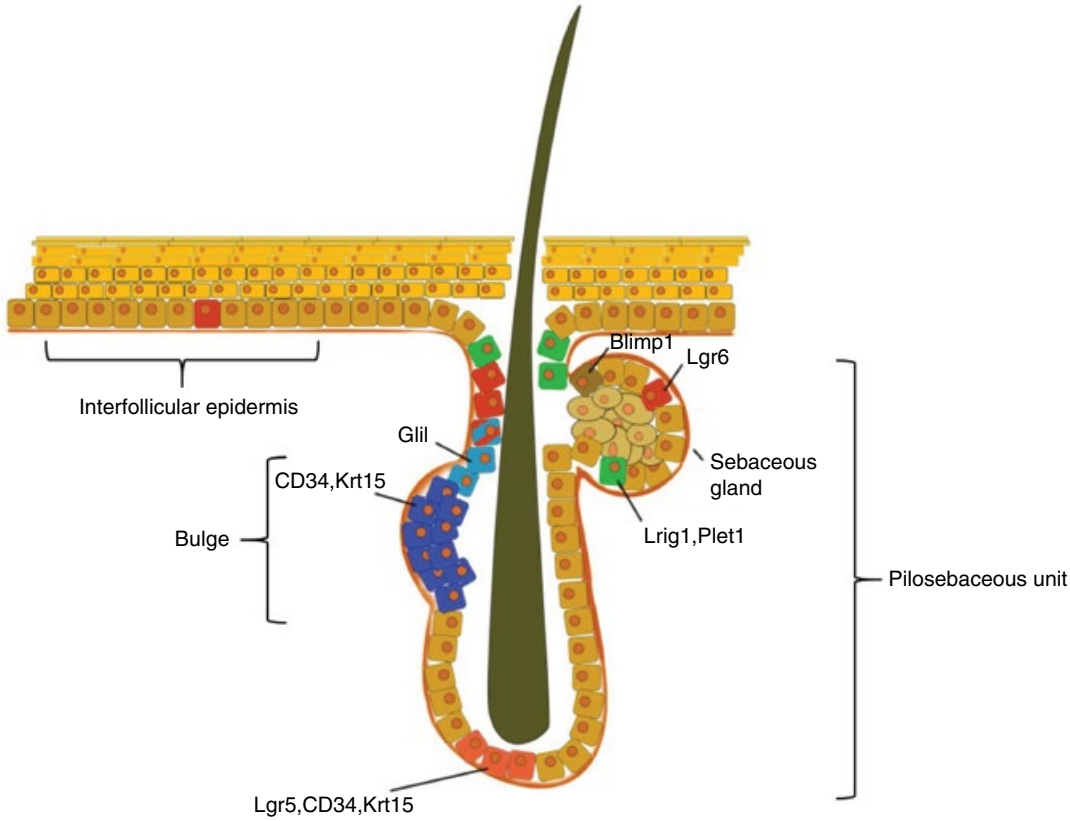

Figure 25.1 ***(Plate 29)*** *Distribution and the biological markers of skin stem cells. (See insert for color representation of the figure.)*

and Lhx2. Lgr6+ cells can generate sebaceous gland and interfollicular epidermis, whereas contribution to hair lineages gradually diminishes with age. Most importantly, Lgr6+ HFSCs also contribute to wound repair.

Interfollicular epidermal stem cells (IFE SCs) are a long-lived, slow-cycling, and highly proliferative stem cell population located in the basal layer of the interfollicular epidermis (IFE). Since the discovery of murine IFE SCs identified *in situ* via cell kinetic studies, it has been a great challenge for scientists to identify the cell surface markers of murine IFE SCs; the identification of human IFE SCs particularly has been severely hampered given that, for ethical reasons, one cannot generate LRCs in humans. The first *in vitro* characterized marker of human IFE stem cells was high expression of ß1 integrin [31], the identified IFE stem cells located in clusters in human IFE *in vivo* [32]. Other studies also reported the expression of a6 integrin and low levels of the transferrin receptor (CD71) [33] and desmoglein-3 (DSG3) [34]. Using mouse lineage tracing and quantitative clonal analyses, it has been found that the Wnt target gene Axin2 is the major biological marker of interfollicular epidermal stem cells. These Axin2-expressing cells constitute the majority of the basal epidermal layer, compete neutrally, and require Wnt/ß-catenin signaling to proliferate [35]. Other candidate markers of IFE stem cells include CD34 and CD117, and it has been found that both IFE SCs and hair follicle SCs do not express CD71 or CD24, suggesting their potential utility as negative selection markers [36].

Sebaceous gland stem cells have been identified with the specific expression of the transcription factor Blimp1. Other studies also detected GPR39 as a specific cell surface marker, which co-localized with another

sebaceous gland stem cell marker, Blimp1. Their study showed that GPR39 was spatiotemporally expressed during skin wound repair and was dispensable for skin development and homeostasis. GPR39 contributed positively to skin wound healing, loss of its expression led to a delay in wound healing [37].

Human mesenchymal stem cells (MSCs) are a heterogeneous population of fibroblast-like cells, which are present in different locations, including bone marrow, adipose tissue, extra-fetal tissues, gingiva, and dermis. MSCs have the capacity for self-renewal as well as potential multipotency. Because of their important expansive potential and immunotolerance properties, MSCs remain an attractive tool for tissue repair and regenerative medicine. Adipose tissue is one of the richest sources of mesenchymal stem cells. Adipose-derived stem cells (ASCs) show outstanding ability in skin repair and wound healing. ASCs express similar surface markers to mesenchymal stem cells, such as CD10, CD13, CD29, CD44, CD54, CD71, CD90, CD105, CD106, CD117, and STRO-1. They are negative for the hematopoietic lineage markers CD45, CD14, CD16, CD56, CD61, CD62E, CD104, and CD106, for the endothelial cell (EC) markers CD31, CD144, and von Willebrand factor.

25.4 Skin Stem Cell Niches

The microenvironment is very critical for stem cell survival and development. Stem cell niche refers to a microenvironment, within the specific anatomic location where stem cells are found, which interacts with stem cells to regulate cell fate. The component of skin stem cell niche includes extracellular matrix, immune cells, fibroblasts, peripheral nerves cells, cutaneous blood vessels, and even progeny of stem cells. Different cell types might influence each other by secreting soluble biological factors or via direct cell interaction; these can help to regulate self-renewal, proliferation, and differentiation of skin stem cells.

The skin consists of multiple types of stem cells consistent with these structural features, it also accommodates a variety of stem cell niches (Fig. 25.2/Plate 30). The dermal papilla is a stem cell niche for mesenchymal stem cells (MSCs) that initiate hair follicle growth [38]. The superior bulge is a stem cell niche for HFSC and melanocyte stem cells (MSCs) [39]. The interaction of the epidermal stem cells with the basement membrane is one important feature in maintaining self-renewal capacity. Basal epidermal cells adhere to the basement membrane through receptors known as integrins. The transit-amplifying cells carrying cell surface makers such as integrinβ1 and higher levels of Ki67 are the immediate progeny of epidermal stem cells; these cells may rapidly proliferate before undergoing the cell differentiation [40, 41]. Therefore, the altered expression of integrin on the cell surface suggests that the interaction with basement membrane maintained the stemness of epidermal stem cells. Connelly's group observed that a decrease of extra cellular matrix may change epidermal stem cells from an expanded shape to a round shape when these cells undergo cell differentiation and this process is controlled by F-actin mediated of serum response factor (SRF) transcriptional activity [42]. Additionally, the stiffness of the cell matrix can also influence stem cell fate through the extracellular-signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway [43] or p38 MAPK and histone acetylation [44].

The skin stem cell niche keeps both the stem cells in quiescence and promotes their proliferation and differentiation. Integrin α3β1 and α6β4 of the integrin family were found to be the functional important molecules that epidermal stem cells adopted to anchor them to their ligand laminin-5 on the basal membrane. In the human basal epidermis, the epidermal cells with higher level expression of β1 integrin show a relatively slow-cycling feature and have a higher colony forming efficiency when plated in culture, suggesting that proliferation capacity is regulated by related cell matrix [45, 46]. Deletion of α6 or β4 integrin, or their ECM ligand laminin-5, leads to severe blistering of epidermolysis bullosao [47, 48], and the signal from α6β4 to Rac1 prevents the epidermis undergoing hyperproliferation to maintain the epidermal stem cell pool [49].

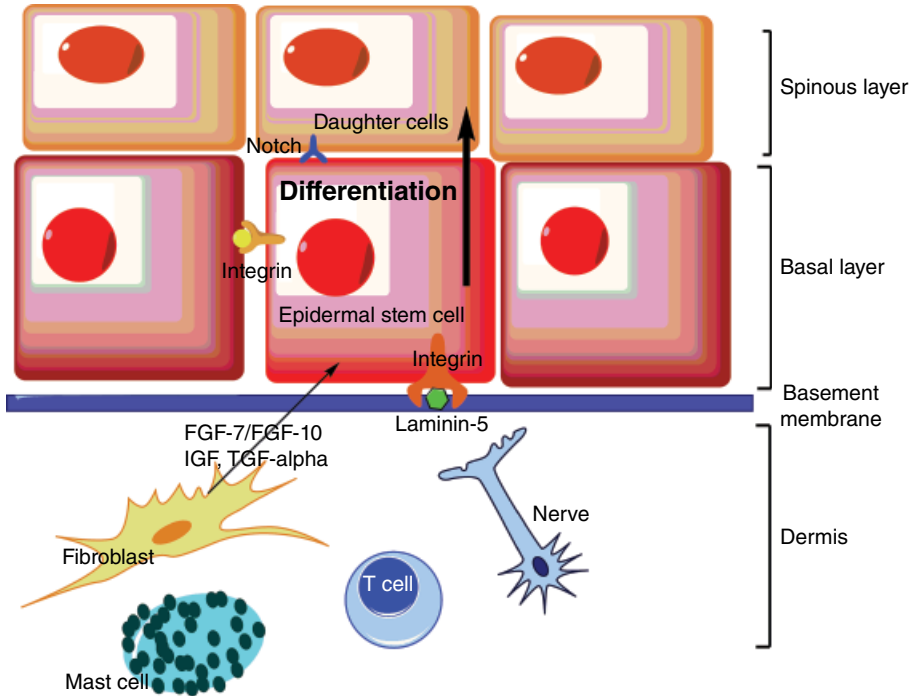

Figure 25.2 ***(Plate 30)*** *Model of skin stem cell niches. (See insert for color representation of the figure.)*

Moreover, the basement membrane alone still does not appear to be the sole component of the stem cell niche. Different types of the cell residing at epidermis also have great influence on the regulation of epidermal stem cell fate. Dermal fibroblasts are a rich source of mitogens such as insulin-like growth factors (IGFs), fibroblast growth factor-7 (FGF-7), FGF-10, and epidermal growth factor receptor (EGFR) ligands, thus facilitating the colony formation of keratinocytes in *in vitro* assays [50, 51]. Activation of transforming growth factor-α (TGF-α), a positive autocrine regulator of EGFR signaling in the epidermis, or inhibition of Mig6, a negative regulator of EGFR signaling in the epidermis, may also cause epidermal hyperproliferation [52, 53].

Stem cell niches also contribute to wound healing after a skin wound occurs; the epidermal stem cells must respond rapidly to restore the compromised barrier and repair tissue damage. This complicated process is accomplished by the interaction of multiple type cells in stem cell niches. Wound healing normally involves three overlapping phases: the initiation of skin inflammation, renewal tissue formation, and regenerated tissue remodeling [54]. The initiation phase is started by various types of immune cells, such as epidermal γδ T cells and Langerhans cells; these cells respond rapidly to the wound, which results in other inflammatory cells being recruited to the injury site. The inflammatory responses normally involved in the secretion of multiple cell growth factors, including FGF-7, FGF-10, and IGF-1 from epidermal immune cells play a key role in angiogenesis, migration, and proliferation of keratinocytes and dermal fibroblasts, synthesis of ECMs, and sometimes generation of new hair follicles in the process of epidermal regeneration [55–57]. NF-kB may be activated by 120-catenin to induce inflammatory responses, then cause epidermal cell hyperplasia in the skin [58].

Adipocyte precursor cells are activated at the tissue formation stage to generate mature adipocytes important for fibroblast recruitment [59]. During tissue remodeling, the epidermis and dermal fibroblasts deposit new ECM proteins to strengthen the repaired tissue. In addition to immune cells, sensory nerves are anatomically in close contact with cutaneous cells in the epidermis and hair follicles. They may influence hair follicle

regeneration. Premature hair follicle regression is elicited by the neuropeptide substances P and CGRP, which trigger neurogenic inflammation [60]. Furthermore, peripheral nerves that innervate the cells above the bulge secrete SHH may regulate cell function during the wound healing process [61].

25.5 Signaling Control of Stem Cell Differentiation

Human skin is able to self-repair throughout an individual's life and this function relies on skin stem cells. Scientific evidence suggests that there are multiple subtypes of skin stem cells that are responsible for replacing the differentiated cells of the interfollicular epidermis, hair follicles, and sebaceous glands. In recent years, considerable progress has been achieved in identifying the signaling pathways regulating skin stem cell decisions. Several evolutionary conserved pathways have been shown to be important for skin stem cell maintenance, differentiation, and lineage commitment including the Notch, Wnt/β-catenin, and MAPK pathways.

25.5.1 Wnt Signaling Pathway

The Wnt signaling pathway is an ancient and evolutionarily conserved pathway that regulates crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning, and organogenesis during embryonic development. Most recently, this pathway has been implicated in stem cell renewal [62]. The Wnt pathway is first aroused in the simplest multicellular organisms in which Wnts act as primordial symmetry-breaking signals, crucial for the generation of patterned tissues during embryogenesis. In adult tissue, the Wnt pathway also controls tissue regeneration. If the Wnt pathway is inhibited, tissue renewal will be crippled. Three signaling pathways are typically described for Wnt proteins. The canonical Wnt-β-catenin pathway (Fig. 25.3/Plate 31), through which ß-catenin-dependent activity occurs; noncanonical pathways, which include the polar cell polarity pathway that involves activation of AP1 through c-jun Nterminal kinase; and the Wnt-Ca^{2+} pathway, which activates protein kinase C and affects cell adhesion [63]. Wnt molecules are highly conserved, cysteine-rich glycoproteins. They function as morphogens that are secreted from certain cell and will bind to the N-terminal extra-cellular cysteine-rich domain of the Frizzled (Fz) receptor, which is a seven trans-membrane span protein with topological homology to the G-protein coupled receptor family. There are 10 Fz proteins existing in humans. The binding of the Fz receptor to the Wnt ligand is involved in both canonical and noncanonical Wnt signaling. In the unstimulated canonical Wnt signaling pathway, β-catenin is phosphorylated by a GSK-3/APC/Axin complex resulting in the ubiquitin mediated degradation of cytosolic beta catenin. Binding of the Wnt ligand to the Fz receptor, complexed with LRP, results in the inhibition of the GSK-3/APC/Axin complex. The accumulated cytosolic beta catenin is further translocated into the nucleus and acts as a cotranscriptional activator of T-cell factor (TCF) and lymphoid enhancer factor (LEF) regulating downstream target genes such as cyclin D1, MMP7, and C-Myc. In noncanonical pathways, signaling is conducted independently of beta catenin. Other Wnt signaling pathways (noncanonical Wnt signaling pathways) include the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway, which is conducted independently of beta catenin, although similar components may be upstream of the Fz receptors. The activity of different Wnt proteins is dependent on the receptor context, which makes strict classification of Wnts into canonical or noncanonical very challenging [63].

Wnt signaling controls stem cell maintenance and fate decisions in the skin and therefore plays a decisive role in multiple steps during skin development and regeneration. Using lineage tracing and quantitative clonal analyses, it is noted that most basal layer cells transduce Wnt signals; these cells can be visualized by the reporter gene assay of Wnt target gene Axin2. These cells are qualified as the interfollicular epidermis (IFE) stem cells, since these Axin2+ basal cells continuously produce keratinocytes for over 1 year *in vivo*, the differentiated keratinocytes are shed from the surface, and these cells contribute robustly to wound healing.

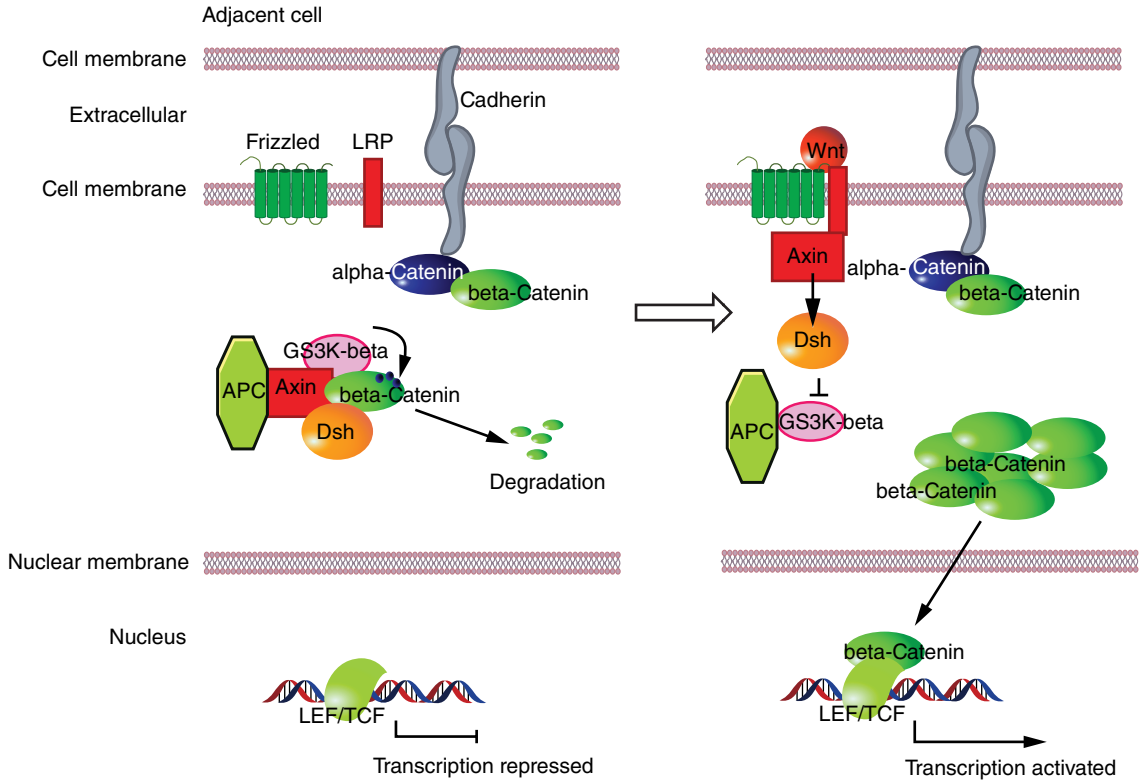

Figure 25.3 ***(Plate 31)*** *Wnt/β-catenin signaling pathway. (See insert for color representation of the figure.)*

There is multiple evidence to suggest that β-catenin is crucial for epidermal proliferation and maintenance of IFE stem cells, both *in vivo* as well as in cell culture. Activation of ß-catenin dependent Wnt signaling has been described as required for the Axin2-expressing cell proliferation. These cells themselves produce Wnt signals as well as long-range secreted Wnt inhibitors, suggesting an autocrine mechanism of stem cell self-renewal [64]. In hair follicle stem cells, hair follicle development also relies on Wnt signaling. Wnt signaling has been reported to be involved in hair placode induction, disruption of ß-catenin in mouse skin epithelium, and dermis impairs the initiation of hair follicle development. Defective hair follicle development is observed in transgenic mice with ectopic expression of the diffusible Wnt inhibitor Dkk1 [63, 65]; a similar pathological phenotype was also found by ablation of the Wnt-dependent transcription factor Lef1 [66].

25.5.2 MAPK Signaling Pathway

Mitogen-activated protein kinases (MAPK) are protein kinases that are specific to the amino acids serine, threonine, and tyrosine. MAPKs belong to the CMGC (CDK/MAPK/GSK3/CLK) kinase group. MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock, and proinflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis [67]. In mammalian cells, three MAPK families have been clearly characterized: namely classical MAPK (also known as ERK), C-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), and p38 kinase. MAP kinases lie within protein kinase cascades.

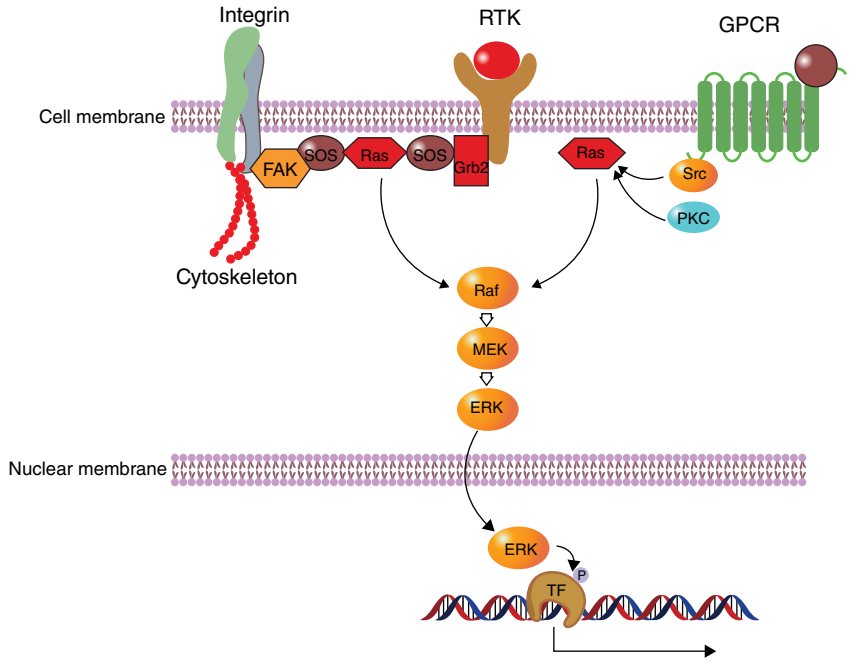

Figure 25.4 ***(Plate 32)*** *MAPK signaling pathway. (See insert for color representation of the figure.)*

Each cascade consists of no fewer than three enzymes that are activated in series: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAP kinase (MAPK). Currently, at least 14 MAPKKKs, 7 MAPKKs, and 12 MAPKs have been identified in mammalian cells [68].

Mitogen-activated protein kinase (MAPK) cascades have been shown to play a key role in transduction extracellular signals to cellular responses (Fig. 25.4/Plate 32). The response of cells to activated MAPK depends on cell type, strength, and duration of signal and on the presence or absence of specific growth factors [69].

The proliferation of epidermal keratinocytes is a crucial step for epidermal renewal and undergoing differentiation. Dermal fibroblasts can produce a bunch of mitogens such as insulin-like growth factors (IGFs), fibroblast growth factor-7 (FGF-7), FGF-1, and epidermal growth factor receptor (EGFR) ligands, and facilitate colony formation of human and mouse keratinocytes *in vitro*. In mouse models, the function of these factors in regulating epidermal proliferation has been well verified. Epidermis lacking insulin-like growth factor receptor (IGFR) is impaired in basal epidermal proliferation [70]. Ectopic expression of mesenchymal factor FGF-7 in epidermal cells causes epidermal hyperproliferation [71]. EGF signaling is a particularly potent pathway for epidermal growth [72]. In mice, activation of transforming growth factor-α (TGF-α), a positive autocrine regulator of EGFR signaling in the epidermis, or deletion of Mig6, a negative regulator of EGFR signaling in the epidermis, leads to epidermal hyperproliferation [53]. In humans, reduction of expression of the EGFR antagonist, LRIG1, promotes human keratinocyte proliferation in culture.

Human epidermal stem cells express higher levels of β1 integrins and are more adhesive than keratinocytes that are destined to differentiate. There are at least three mechanisms by which β1 integrin mediated adhesion can activate MAPK [73]. In the first two, MAPK is activated via Ras, either through FAK or Shc [74,75]. In keratinocytes, FAK phosphorylation was not inhibited by CD8β1, as observed when similar dominant negative β1 constructs are expressed in other cell types [76]. The Shc-mediated pathway involves the interaction

of the transmembrane and juxtamembrane extracellular domains of integrin, and it is difficult to envisage how this process would be affected by overexpression of the β1 cytoplasmic domain. Our data favor a third mechanism that is independent of Ras and FAK. It should also be noted that in some contexts MAPK can downregulate β1 integrin function.

Although Zhu et al. demonstrated a role for the MAPK cascade downstream of β1 integrins in controlling proliferative potential [77], Mainiero et al. have shown that in keratinocytes, α6β4-mediated adhesion results in MAPK activation via Ras and promotes cell-cycle progression in response to mitogens [78], an effect that could probably influence stem and transit-amplifying cells to the same extent because their α6β4 levels are similar and their cell-cycle kinetics are virtually identical [79]. Thus in keratinocytes MAPK is downstream of both the β1 and β4 integrins and can be regulated by distinct pathways with distinct biological consequences. A signaling pathway involving β1 integrins and MAPK controls epidermal stem cell fate *in vitro*. Ligand binding suppresses terminal differentiation within the basal layer of the epidermis [80], high levels expression of cell surface molecules β1 integrins could protect stem cells from differentiation. Because the β1 integrins have a pericellular distribution in stem and in transit amplifying cells [45], the proportion of surface integrins in contact with the basement membrane will be similar in both cell populations. It therefore seems likely that it is the absolute number of occupied receptors that is important for the protective effect [81]. Although epidermal stem cell number is subject to autoregulation, it is generally believed that environmental factors, specifically the composition of the basement membrane, could also be key determinants. Stimulatory or inhibitory input into the β1-integrin MAPK pathway at different levels could provide a mechanism by which the environment influences the proliferative capacity of basal keratinocytes. ECM proteins can modulate β1 integrin expression and activation of the erative capacity of basal keratinocytes. ECM proteins can modulate β1 integrin expression and activation, and local variation in the composition of the basement membrane could thus play a role in establishing and maintaining the patterned distribution of stem cells within the epidermal basal layer.

25.5.3 Notch Signaling Pathway

The Notch signaling pathway is a highly conserved cell signaling system present in most multicellular organisms (Fig. 25.5/Plate 33). Notch signaling defines an evolutionarily ancient cell interaction mechanism, which plays a fundamental role in metazoan development. In the different organs, Notch signaling has been found involved in the regulation of a wide variety of cellular processes, including the maintenance of stem cells, cell fate specification, differentiation, proliferation, and apoptosis [82].

In the vertebrate hematopoietic system, Notch signaling influences the balance between the progenitor cells (including stem cells) and their differentiating cells, by inducing these cells to retain the undifferentiated state. In addition to preserving undifferentiated states, Notch signaling also participates in specification of cell fate. This is most apparent during the development of neuronal precursor cells in *Drosophila*, which originate from a group of equipotent cells that have the capacity to develop into either neuronal precursor cells or epithelial cells. Cells that receive Notch signals are prevented from leading to neuronal development and thus adopt an epithelial cell fate, whereas cells that exclusively express ligands are driven into the neuronal cell fate [31]. Similarly, Notch signaling influences the cell fate choice of precursor cells that ultimately become T- or B-cells during lymphocyte development [32]. Furthermore, Notch signaling can also induce terminal differentiation, an example of which is seen in the epidermis [33].

Skin undergoes self-renewal throughout life. In the human epidermis, Notch1 is expressed in all of the epidermal layers, Notch2 is expressed in the basal layer. In the mouse epidermis, however, Notch1 is expressed most strongly in the spinous layer, and Notch2 is also expressed in the spinous layer but not in the basal layer [83].

Multiple Notch signaling family members are expressed in embryonic and adult epidermis. During the early stage of epidermal stratification, Notch1 is expressed and active in the basal and suprabasal cells of the

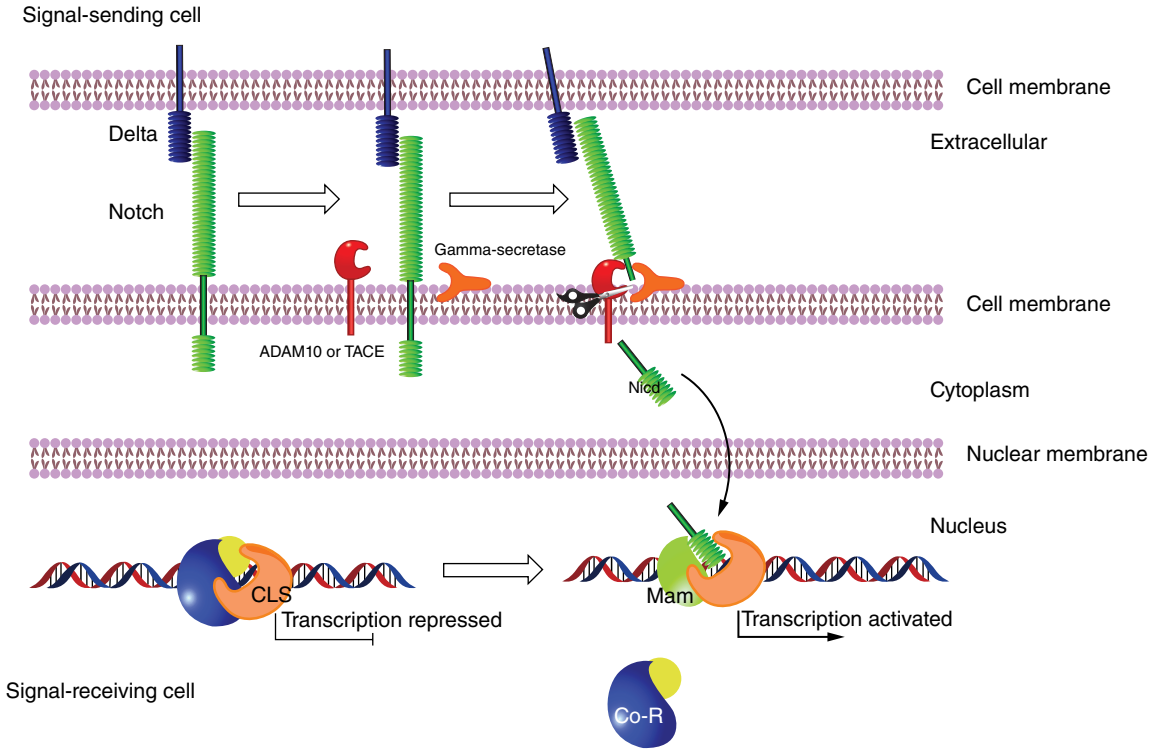

Figure 25.5 ***(Plate 33)*** *Notch signaling pathway. (See insert for color representation of the figure.)*

epidermis and sebaceous glands [84, 85]. In the latter stages of epidermal stratification, Notch1 activity becomes more restricted to the spinous layer. And the distribution of notch ligands in the epidermis are Delta like 1 (Dll1; Delta1) in the basal layer, Jagged1 in the suprabasal layers and some basal cells, and Jagged2 in the basal layer [86, 87]. Deletion of Notch2, Notch3, or Notch4 alone does not have any reported effects on the epidermis [88–90]. But deletion of Notch1 may cause hair follicle morphology destruction and lack of mature sebocytes in adult epidermis [91]. The mechanism by which Notch activation induces IFE terminal differentiation has been examined primarily in keratinocytes. The results show that canonical signaling via RBP-Jk is involved as well as complex interaction with other pathways [92, 93]. Notch1-mediated induction of p21 may cause growth arrest to keratinocytes that undergo terminal differentiation [93]. Notch can also suppress of p63 expression and induced epidermal stem cells differentiation. p63 negatively regulates Hes1 gene expression and counteracts the differentiation-promoting activity of Notch activation, suggesting that a mutual antagonism between Notch and p63 is involved in epidermal homeostasis [94].

25.6 Stem Cells in Skin Aging

The skin constantly renews itself throughout adult life. Skin regeneration is a process of replacement of the surface out-layer by the deeper layer of the skin. This process starts from the deep layer of the dermis and eventually moves to the epidermis relying on epithelium stem cells. This function is very important for the maintenance of adult skin homeostasis and also for wound healing after tissue injury. Skin cells can regenerate

and move to the surface layer within 2 weeks, but this is typically only true for younger skin. Adult skin tissue homoeostasis requires continual replacement of keratinocytes that are lost due to normal turnover, injury, and disease. Skin is maintained through a balance of proliferation, differentiation, and self-renewal of stem cells to keep and repair for organ homeostasis [95, 96].

Skin aging is associated with an overall decline in tissue function and homoeostasis. Skin changes are among the most visible signs of aging; the common signs of skin aging include wrinkles and sagging skin. Skin aging is an inevitable part of aging due to the accumulation of ROS damage to mitochondria, the loss of proteostasis, and so on, which alter the equilibrium resulting in altered numbers, decreased organ function, and an inability to tolerate stress [97]. Within the skin, aging is associated with a loss of fibrous tissue, slower rate of cellular renewal, and a reduced vascular and glandular network. Although the number of cell layers remains stable, the skin progressively becomes thinner at an accelerating rate throughout adult life [98]. The aging of skin cells may result in a progressive decrease of turnover epidermal time and loss of tone and elasticity of skin [99, 100]. One of the most import features of aged skin is the reduction in skin renewal ability compared with younger skin. This contributes to wrinkles, sagging, and slower wound healing in which collagen, elastic fiber, and stem cells play important roles [101]. Recently, it has been indicated that dysfunction or loss of certain skin-resident stem cells in the premature aging of skin may contribute to impaired skin functions, which included a defect in skin renewal capacity, reduced wound healing, and the development of diverse skin disorders, such as an increased susceptibility to injury and infection, epidermal dehydration, wrinkles, pigmentary alterations, hair graying and loss, and an increased risk of skin epithelial cancers and melanoma [102]. It is generally acknowledged that epidermal stem cells exhibit decreased colony-forming ability and delay of activation [103]. Giangreco et al. reported that during the human skin aging process, there is reduced expression of stem cell markers such as β1 integrin and MCSP [104], indicating that in the case of chronologic aging, the levels of epidermal stem cells tend to decrease and change functionally.

Apart from the interfollicular epidermal stem cells, hair follicle stem cells are susceptible to age-associated changes that may contribute to aging phenotypes [105]. Keratin-15 positive hair stem cells were proved to be one of the best-marked populations in skin with multipotent capacity, contributing to hair follicle cycling, sebaceous gland function, and wound repair. Although aging stem cells showed high levels of keratin-15 expression, when co-expressed with CD 34, their ability to form a hair germ and to proliferate is reduced [106]. Another marker Lgr5, p16INK4a/Arf, a senescence marker, accumulated in aged skins [107].

The pathological mechanism of skin aging currently still needs to be defined. Current knowledge indicates that oxidative stress (OS), ultraviolet radiation exposure, telomere shortening in epidermal stem cells, hormone exhaustion, and genetic events that result in severe DNA damage, genomic instability, and epigenetic mutations are the likely major contributors to the induction of skin aging [108]. Environmental stimuli, for example exposure to ultraviolet (UV) light, could trigger skin aging by inducing photooxidative damage; Other factors, such as cigarette smoke, pollutants, and the natural aging process also cause skin aging by generation of free radicals and ROS [109, 110]. During the skin aging process, although there is no difference regarding the cell numbers and gene expression in between young and aged epidermal stem cells, the latter shows reduced expression of antioxidant enzymes such as superoxide dismutase (SOD), an enzyme required to catalyze the dismutation of superoxide. In animal studies, the genetic knock-out of the superoxide dismutase (SOD) gene in the skin results in increased skin aging and a decreased lifespan in the mutant mice, indicating that OS might change the physiological potential of skin stem cell via new epigenetic programming [111].

Sunlight exposure is healthy for the skin because it provides vitamin D, but an overdose of sunlight exposure can damage the skin irreversibly. Photooxidative damage is a process triggered by the sunlight dependent generation of reactive oxygen species in the skin. In young healthy skin, the generated ROS are eliminated

rapidly by efficient cutaneous antioxidative systems. However, in aged skin, the overwhelming production of ROS normally initiates DNA damage, leading to the formation of thymidine dimers.

Hormone exhaustion is another cause of skin aging; it is well known that estrogen can prevent collagen loss, increase skin thickness, restore skin moisture, prevent hair loss, and promote wound healing, and these functions rely on the recognition and binding of estrogen with estrogen receptors (ERs), which are widely expressed in epidermal keratinocytes, dermal fibroblasts, blood vessels, melanocytes, and dermal MSCs. Epidermal stem cells also express ERs, since the expression of ERs has been documented in hair follicles, dermal papilla cells, and the bulge region of outer root sheath where they may regulate hair growth directly via those cells [108]. It is well known that levels of estrogens rapidly decline in menopause; the deduced exposure of stem cells to estrogen in both dermis and the epidermis thus causes a decline in the number of fibroblasts and in collagen production, as well as diminished elasticity and strength of skin [112].

Telomere shortening is another mechanism causing skin aging. In skin, the expression of telomerase can be detected only in certain skin stem cell components, for example in cells of the basal epidermis and in the bulge component of the hair follicle [113]. Skin stem cells are sensitive to telomerase dysfunction. In a mouse study, it has been demonstrated that skin homeostasis is defective when telomeres are TERT dysfunctional [114]. Epidermal stem cells isolated from telomerase deficient mice exhibited a low proliferative capacity in *ex vivo* cell culture [115].

Skin stem cells are frequently exposed to various kinds of DNA damage and increased accumulation of DNA damage is associated with skin stem cell senescence and skin aging. Physiologically, hair follicle stem cells have two important mechanisms for resisting DNA damage, higher expression of the anti-apoptotic gene Bcl-2, and transient stabilization of p53. The abnormal regulation of molecular signing of these can directly damage skin homeostasis [116]. In a separate study, it has been demonstrated that deletion of Mdm2 gene, the chief negative regulator of p53, in mouse skin could induce an aging phenotype, including thinning of the epidermis, reduced wound healing, and a progressive loss of fur [117], which further supports the critical role for maintaining DNA integrity in regulating skin homeostasis and aging.

25.7 Stem Cells in Skin Cancer

Although the idea that a tumor grows following a signal cell is accepted by most people, it shows a highly heterogeneity feature, suggesting the existence of multipotential cells in tumors, especially while they accomplish resistance change to certain treatments. The idea of cancerous stem cells is not a new one; Hamburger and Salmon proposed this concept in 1977 [118]. This concept tried to explain the heterogeneity of tumor cells through nongenetic mechanisms, but at that time the insufficiency of cell sorting and molecular techniques made it impossible to isolate and identify a subpopulation of tumor cells. So, this concept cannot be fully accepted due to the shortage of experimental evidence.

In the 1990s, researchers started to discover that a subpopulation of tumor cells with certain cell surface markers could give rise to a new tumor at a low cell number [119]. They named these cells cancer stem cells or tumor progenitor cells. These cells are less than 1% of the tumor number but can give rise to a new tumor sphere. Most of the early work on cancer stem cells was based on the FACS technique. After sorting a subpopulation of tumor cells, a few cells (even a single cell) were injected into immune deficient mice and the formation of new tumor was observed. These findings are observed in breast cancer, AMLs, lung cancers, prostate cancer, glioma, skin cancers, and so on. But it is still a concern regarding whether this xenograft assay can truly reflect the actual fate of the tumor formation from which cells are originally derived, since there are many environmental variables in the tumor environment, such as hypoxia and immune responses that can prevent the so called “cancer stem cells,” forming a tumor in their normal

physical environment. Stem cells require certain regulatory factors maintained in their niche to keep stemness, as do cancer stem cells. The niche of cancer stem cells is also critical for keeping their quiescent and undifferentiated state and also for maintaining their proliferation and differentiation potentials. The non-epithelial stromal cells in the microenvironment of cancer stem cells may help in the nursing and training of cancer stem cells through direct contact or secreted factors. The so called tumor associated stromal cells include inflammatory cells, vascular endothelial cells, and fibroblasts. In the case of squamous cell carcinoma, the crosstalk of BMP family members secreted by tumor cells and GREMLIN1 and FOLLISTATIN expressed by tumor associated stromal cells are essential for the self-renewal of cancer stem cells. Furthermore, the VEGF–Nrp1 loop also helps the formation of the vascular niche and regulates the initiation and stemness of epithelial tumors. So, this fact indicates that targeting these tumors associated stromal cell may be a reasonable therapeutic strategy for cancer treatment, since directly targeting tumor cells is relatively difficult.

Like normal stem cells, cancer stem cells possess self-renewal capacity; however, self-renewal is typically disregulated. Normally, the amount of cancer stem cells is rare, but in some types of tumor it has not been possible to distinguish CSCs from non-CSCs because most cells have some CSC function. Such tumors seem to be homogeneous or possess a very shallow hierarchy. Thus, it is possible that cancer cells have plasticity by reversibly transitioning between a stem and non-stem-cell state. Today's most important method is based on FACS, so the cell surface markers of cancer stem cells are critical. However, cancer stem cells also show great heterogeneity on cell surface markers. Nonetheless, a number of markers have proven useful for the isolation of subsets enriched for CSCs in multiple types of solid tumors, including CD133, CD44, EpCAM, and ALDH activity.

In the case of skin cancers, CD 20+, ABCB5 is considered to be a marker of melanoma and CD34 for squamous cell carcinomas. But in some cases it has proven difficult to confirm when these markers originally appeared to robustly distinguish tumorigenic from non-tumorigenic cells, especially with one marker. Therefore, the combination of several surface markers simultaneously or screening with functional approaches may give a better result. So far, the signaling pathways of skin cancer stem cells are not quite clear, but there are several important studies demonstrating the factors controlling skin stem cell self-renewal, initiation, and proliferation. POSTN on tumor associated fibroblasts may regulate metastatic colonization through Wnt signaling. Boumahdi's group showed that Ras upregulated SOX2 and SOX2 are involved in multiple processes of tumor development such as proliferation, survival, and stemness maintenance [120]. CD151 may promote SCC through the activation of STAT3. Furthermore, TGF-β, integrin/focal adhesion kinase (FAK) signaling, and β-catenin pathways are all shown to be vital for the regulation of skin cancer stem cells.

The critical role of skin stem cells in the control of skin tumor development has been demonstrated. In one study, by using an abrasion technique to remove interfollicular epidermis and leave the hair follicles undisturbed, Morris et al. demonstrated that mice in which the interfollicular epidermis had been removed still developed papillomas and carcinomas; however, the number of papilloma was only half that of the unabraded mice. Their results indicated that the targets of skin tumor initiation are skin stem cells found in the hair follicles and, to a lesser degree, in the interfollicular epidermis [121]. Another study by Malanchi et al. also demonstrated that skin cancer stem cells (SCSCs) derived from a CD34+ cell population were characterized by phenotypic and functional similarities to normal bulge skin stem cells. This CD34+ cell population contains SCSCs, which are the only cells with tumor initiation properties. Ablation of the β-catenin gene results in the loss of SCSCs and complete tumor regression [122]. Apart from skin stem cells, some epidemiological data and recent findings also demonstrated that skin cancers could also originate from bone marrow-derived or other extra-cutaneous stem cells in addition to local stem cells. These ideas have support from a study on the role of bone-marrow derived inflammatory cells in the development and progression of SCCs in the K14-HPV16 transgenic mouse model and

one of the crucial ideas derived from this article reveals that bone marrow-derived cells very likely participate in SCC carcinogenesis developments [123].

25.8 Medical Applications of Skin Stem Cells

25.8.1 Stem Cells in Tissue Engineering and Skin Repair

Tissue engineering is a research field that uses a combination of methods to engineer cells and biomaterials to improve or replace the biological functions of human tissues. Human skin has a number of vital protective and homeostatic functions. Since skin is exposed directly to the external environment, it frequently encounters various kinds of environmental stimuli. Although skin is well recognized as the largest organ of the body, it is rarely seen as a vital one like the brain or the heart. However, destruction of just 15% of the skin's total body surface area is sufficient to be life-threatening. Engineered skin substitutes have attracted great scientific attention due to their critical value in medical applications for the treatment of large scale of skin loss or damage.

Early skin substitutes used cultured epithelial sheets. This is routinely used to make autologous grafts and this approach can be lifesaving for treating patients with a large percentage of skin loss [124]. These skin autografts or allografts serve as a cellular dressing. The limitations of this approach are pain and scar formation at the donor site; moreover, other disadvantages also include impaired wound healing and non-healing wounds, insufficient material to cover a large defective area, and autoimmune rejection in the case of an allograft [125]. Following these early studies, research that followed placed effort on the generation of engineered epidermis using keratinocyte and fibroblast seed cells isolated from the skin; however, culture of these autologous cells takes time, which limits their usefulness, and donor site insufficiency is a problem for patients with large skin defects [126].

The second generation of bioengineered skin has emerged by combination of stem cells with specifically designed novel biomaterials. The principles of this approach are to initiate cell cultures *in vitro*, grow them on scaffolds *in situ*, and transplant the composite into a recipient *in vivo*. One study by Steffens et al. has developed a cutaneous substitute by bringing together mesenchymal stem cells, keratinocytes, and an electrospun biomaterial, and this cutaneous substitute has been demonstrated with similar skin tissue structures [127]. Another report by Limat et al. generated epidermal equivalents from cultured hair follicle stem cells and used them for the treatment of patients suffering from recalcitrant chronic leg ulcers. The results were found to be very successful since five out of seven ulcers completely healed within 2 weeks after they received the grafted epidermal equivalent treatment [128]. In another study, direct graft transplants of hair follicles containing autologous scalp cells were tested in chronic leg ulcers, and the potential healing capacity of this treatment was assessed. In this clinical pilot study, 10 patients with ulcers of an average size of 36.8 over a 10.5-year duration were enrolled in an 18-week observation. There was a significant reduction in ulcer area in the experimental square compared with the control square (27.1% vs 6.5%) and this result indicated that terminal hair follicle grafting into wound beds represents a promising therapeutic alternative for nonhealing chronic leg ulcers.

Stem cell based 3D bioprinting technology has emerged and might represent a future direction for human skin tissue engineering. Bioprinting technology is a fabrication technology used to precisely dispense cell-laden biomaterials for the construction of complex 3D functional living tissues or artificial organs. Although still immature, bioprinting strategies have demonstrated their potential use in regenerative medicine to generate a variety of transplantable tissues, including skin, cartilage, and bone. Current bioprinting approaches still present technical challenges in terms of high-resolution cell deposition, controlled cell distributions, vascularization, and innervation within complex 3D tissues. However, in the near future, by combination with the

recent advances in human stem cell technologies, 3D bioprinting technology might provide a new challenge for broad biomedical engineering applications [129].

25.8.2 Stem Cells in Hair Follicle Regeneration

Hair loss, which is common seen both under physiological and pathological conditions, may have profound emotional and psychological effects on patients. Hair loss has multiple potential causes, including diet, mineral deficiency, medication, severe stress or illness, pollution, injuries, infection, or skin pathologies; however, reconstitution of a fully organized and functional hair follicle from dissociated skin stem cells is a challenge still pending in tissue engineering. Different strategies aiming to regenerate or neogenerate the hair follicle have been tested. Among these strategies, neogenesis of hair follicles by tissue engineering has emerged and might be applicable for clinical uses in the near future.

Tissue engineering based follicle neogenesis means reconstitution of dissociated epidermal stem cells and inducing dermal cells to form hair follicles. The major principles of this approach include 3D cell culture conditions generated by the cells themselves or by the use of biocompatible scaffolds [130]. Scientists are currently working to engineer hair follicles that require hair forming competent epidermal cells and hair inducing dermal cells. Significant progress has been achieved in animal models that lead to hair formation.

Zheng et al. described a system for rapidly and reproducibly generating hair follicles from dissociated epithelial and mesenchymal cells. In their study, hair follicle growth was observed when dissociated skin progenitor cells isolated from newborn mouse skin were injected into adult mouse truncal skin [131]. Lee et al. developed a simplified procedure to regenerate hair producing skin. By mixed cultured of the epidermal and dermal cells from newborn mice in different ratios, they seeded the cells on the collagen side of the IntegraTM matrix, the result constructs were grafted in full thickness skin wounds generated on the back of athymic mice. Then 8 days after grafting, a hair germ started to appear and progressed to a hair peg, which gradually developed into complete hair follicles 4 days later, and these hair follicles have been found to maintain the ability to continuously cycle for more than 1 year. These reports described a useful approach for regeneration of hair follicles with specific sizes and shapes for the treatment of alopecia by tissue implants [132].

25.8.3 Stem Cells in Wound Healing

Normal wound healing is a complex and well-orchestrated process involving complex interactions among cells, growth factors, and extracellular matrix (ECM) molecules to sequentially achieve homeostasis, cell proliferation, angiogenesis, re-epithelialization, and remodeling of tissue. Impaired wound healing, such as diabetic ulcers and arterial ulcers, remains a challenge to date and causes debilitating effects with tremendous suffering. Compared with other traditional treatment approaches, such as direct cytokine and growth factor treatment, whose disadvantages include low stability and short *in vivo* half-life, cell therapies offer a huge potential in the field of cutaneous wound healing. Apart from the improvements in *in vivo* delivery efficiency, live cell therapy can reduce the potential risk of growth factor therapy, for example the delivery of platelet derived growth factor (PDGF) may increase incidences of cancer mortality [133].

Stem cells have been reported to be directly involved in normal tissue repair/regeneration during wound healing. In the skin, upon wounding, multiple stem cell populations in the hair follicle (HF) and interfollicular epidermis (IFE) converge at the site of injury and contribute to wound healing. Apart from their physiological role in controlling wound healing, skin stem cells have attracted great attention in the treatment of nonhealing wounds. Among these studies, adult stem cells have been most widely used as they are relatively easy to obtain and culture. Different to embryonic stem cells, which raise many ethical concerns, adult stem cells can be obtained from almost any tissue. In recent years, stem cells have been extensively assessed for their wound healing potential in both preclinical and clinical settings, mainly in critical limb ischemia and diabetic wounds.

Over the last few years, several different types of stem cells have been studied in both preclinical and clinical settings such as bone marrow-derived mesenchymal stem cells (MSCs), adipose-derived stem cells (ASCs), circulating angiogenic cells (e.g., endothelial progenitor cells), human dermal fibroblasts, and keratinocytes for wound healing.

Mesenchymal stem cells (MSCs) are multipotent stem cells and these cells are capable to repair not only mesenchymal tissues (bone, cartilage, muscle, marrow, tendon, and ligament) but also liver, heart, nervous tissue, and skin [134]. MSCs exhibit site specific differentiation, responding to environmental cues and adapting their functions to diverse biomolecular contexts [135]. With the specific capacity for stimulating angiogenesis, reducing local inflammation, and promoting the formation of the extracellular matrix, MSCs have been most widely used for the treatment of wound healing [136]. Topical delivery of bone marrow-derived MSCs on a collagen sponge scaffold showed significant improvement in wound healing [137]. In another study, MSCs combined with autologous biografts have proved to efficiently improve wound healing outcomes in chronic diabetic skin wounds [138]. Therefore, stem cell therapy could be a reliable choice for the treatment of hard to heal chronic wounds. Another study also indicated that MSC might functionally improve scar formation. In this study, it was found that MSC elicits leukocyte migration for skin homeostasis and produces hepatocyte growth factor and basic fibroblast growth factor to inhibit scar formation at the wound site [139].

Adipose-derived stem cells (ADSCs) are another widely used type of stem cell for establishing wound healing treatment. ADSCs have a fibroblastic morphology, which consists of a large endoplasmic reticulum and a large nucleus. ADSCs are pluripotent stem cells with the ability to differentiate into different lineages and to secrete paracrine numerous growth factors and cytokines critical in wound healing. ADSCs also increase macrophage recruitment, enhance granulation tissue, and improve vascularization. ADSCs are relatively stable; if necessary, the fresh isolated ADSCs can be cryo-preserved for up to 6 months prior to their therapeutic use in the future. Adipose tissue is a favorable source of stem cells as it can be extracted in large amounts with minor donor site morbidity. Isolation of ADSCs from adipose tissue is easily achieved, which involves digestion of the lipoaspirated tissue with collagenase, accompanied by a subculturing step to detach the ADSCs from the primary adipocytes [140]. Skin wounds treated with ADSCs have shown enhanced healing via epithelial migration, angiogenesis with better healing rates, and less scar formation. ADSCs can also secrete various kinds of cytokines and growth factors. In one of the studies, *in vitro* ADSCs cultured media were analyzed and it was found that the secretion profile of ADSCs included TGF-β, vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), fibroblast growth factor 2 (FGF2), PDGF, HGF, fibronectin, and collagen I. All of these factors have previously been found to benefit improvement in chronic wound healing [141]. In a separate study, it has been found that ADSCs, when combined with different scaffolds, showed improved wound healing. Altman et al. showed that seeded ADSCs on silk suture can close full-thickness skin wounds in mice [142]. In their study, it was noted that wound healing rate was significantly improved in response to ADSC treatment.

ADSCs may also be useful for the treatment of pathological wound healing in the context of aberrant scar formation. The extent of scar formation is closely associated with the inflammatory process in wound healing, ADSCs have been shown to have anti-inflammatory and immunosuppressive effects [143]. A study performed on porcine skin wounds indicated that ADSC treatment could result in better cosmetic results compared to control treatments [144]. Yun et al. injected ADSCs subcutaneously into scars formed from full thickness skin defects on the backs of pigs. Their results showed that scar surface area was significantly smaller in the experimental group, which also showed greater improvements in scar color and pliability [145].

25.9 Conclusions and Future Directions

In this chapter, we have discussed the component and the biological function of skin stem cells. It was identified that there are various types of skin stem cell populations residing in distinct locations in both the skin

epidermis and dermals. The most commonly seen skin stem cell population includes hair follicle stem cells, interfollicular epidermal stem cells, sebaceous stem cells, melanocyte stem cells, dermal mesenchymal stem cells, and adipose-derived stem cells. Each stem cell population retains the capacity to differentiate into cells of all epidermal lineages or other specific cell types. The quiescence, senescence or apoptosis, proliferation, and differentiation of skin stem cell populations are tightly controlled by various intercellular signaling pathways. The most commonly seen signaling pathways include Wnt, MAPK, and Notch among others. There is increasing evidence showing that the altered regulation of adult skin stem cells is associated with numerous skin pathologies. Also, oxidative stress, ultraviolet radiation exposure, or other genetic events that result in severe DNA damage, genomic instability, and epigenetic mutations in skin stem cells contribute to skin aging and skin cancer.

Currently, scientists are putting increased efforts into characterizing the skin stem cell population; these include identifying and isolating pure adult stem cell subsets, optimizing *in vitro* cultured protocols, and exploring their pathological role in skin diseases. The research community strives to elucidate the differentiation potential and biological functions of adult stem cells, associated molecular pathways, complex cellular interactions, and regulators of stem cell behavior in order to restore disturbed skin homeostasis, thus helping the future development of more effective therapeutic strategies for the treatment of various kinds of skin disorders and nonhealing wounds. In the near future, more clinical trials will be performed to further explore the long term effects of using the skin stem cells and to ultimately provide safer and more effective therapies for future clinical applications.

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Author Index

Stem Cells in Toxicology and Medicine, First Edition. Edited by Saura C. Sahu.

Subject Index

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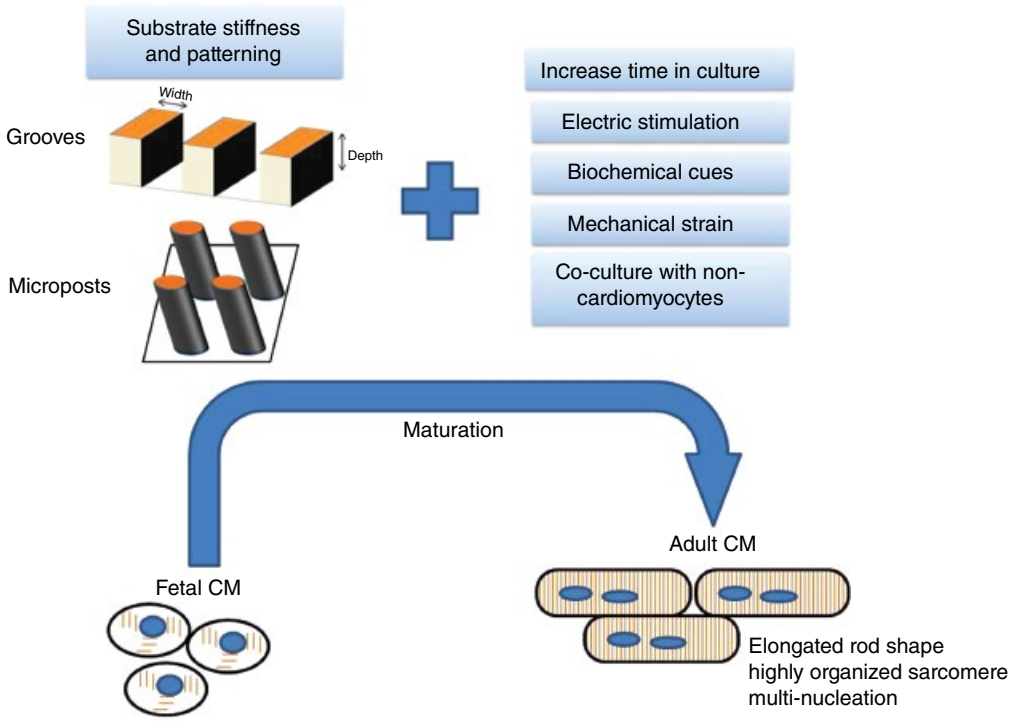

Plate 1 ***(Figure 5.1)*** *Examples of substrate patterns that have been used in an attempt to develop more mature cardiomyocytes. Various patterns in the substrate have been used alone or in conjunction with conditions listed on the right to produce a cardiomyocyte with a phenotype that is more similar to that of an adult cardiomyocyte*

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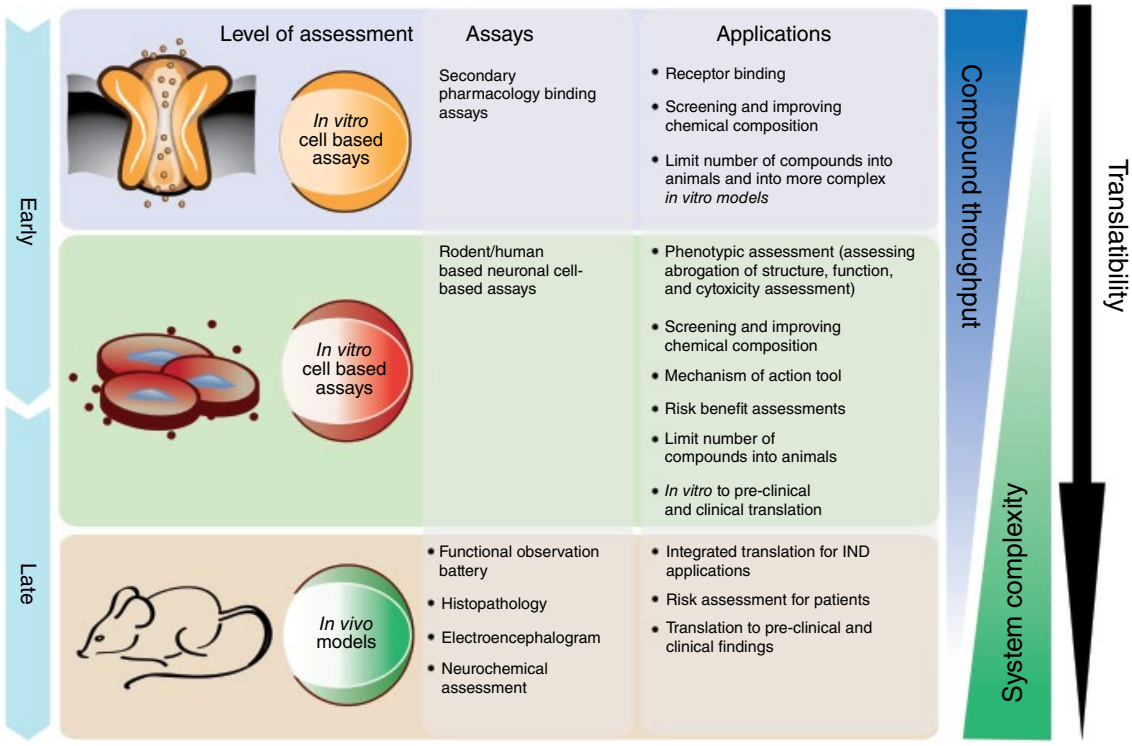

Plate 2 ***(Figure 9.1)*** *High level neurotoxicity evaluation cascade in drug development. Preliminary assessments of the potential for neurotoxicity using simple in vitro tools aim to improve chemical matter and reduce the number of compounds moving through the pipeline as potential drug candidates. Once promising candidates have been selected, additional risks (depending on the target), and the mechanism of toxicity observed both pre-clinically and clinically can be evaluated in neuronal specific cell assays to query functional effects. Ultimately, these neuronal specific in vitro models further reduce the number of compounds that are assessed in animal studies and help to identify the molecular underpinnings of neurotoxicity. This diagram presents approaches conducted both at the early and late phase of drug development process*

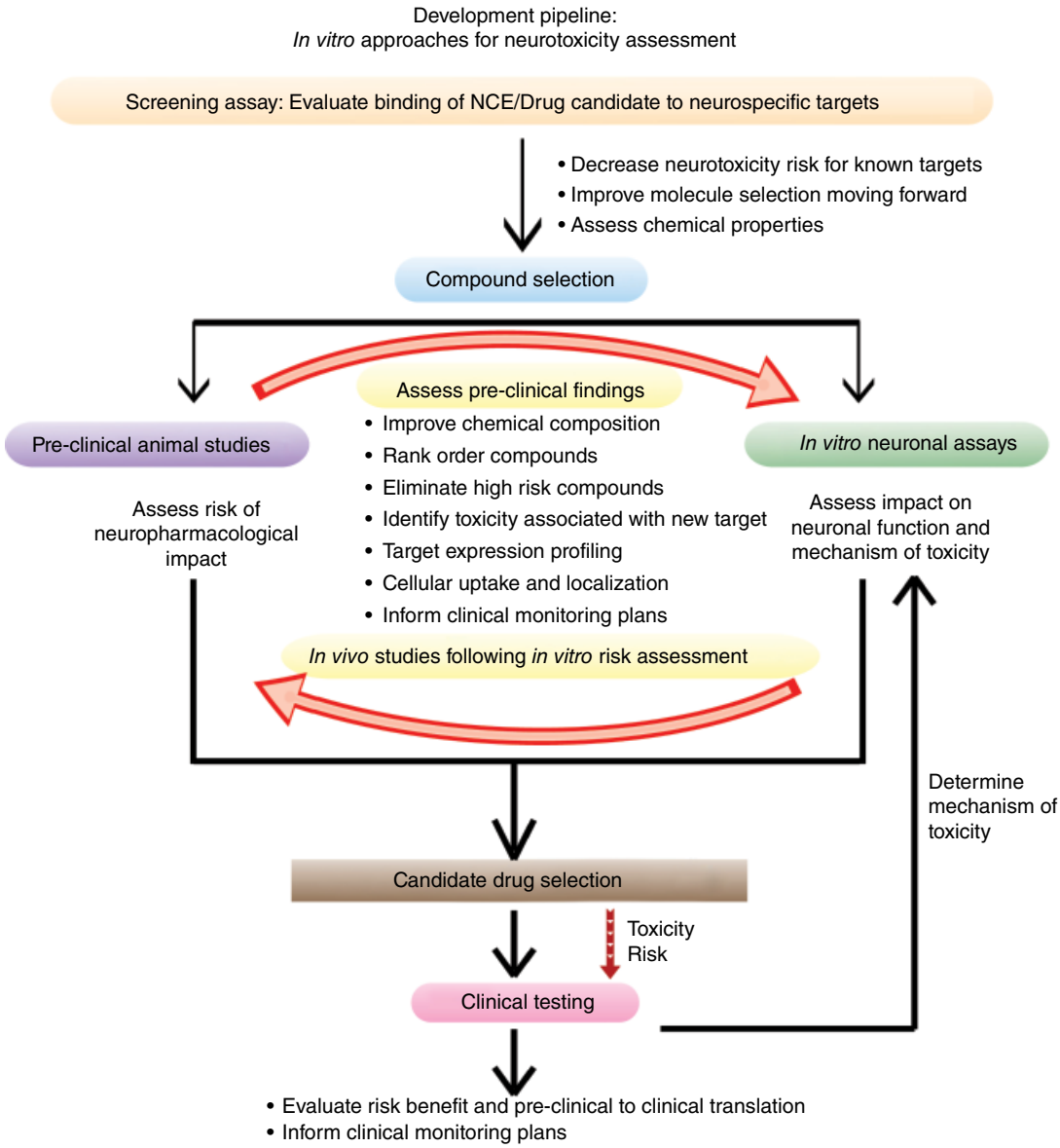

Plate 3 ***(Figure 9.2)*** *Flowchart illustrating the incorporation of* in vitro *approaches for neurotoxicity assessment in the drug development process and highlights the potential impact on drug candidate selection and neurotoxicity risk assessment aiming to reduce the clinical impact on attrition*

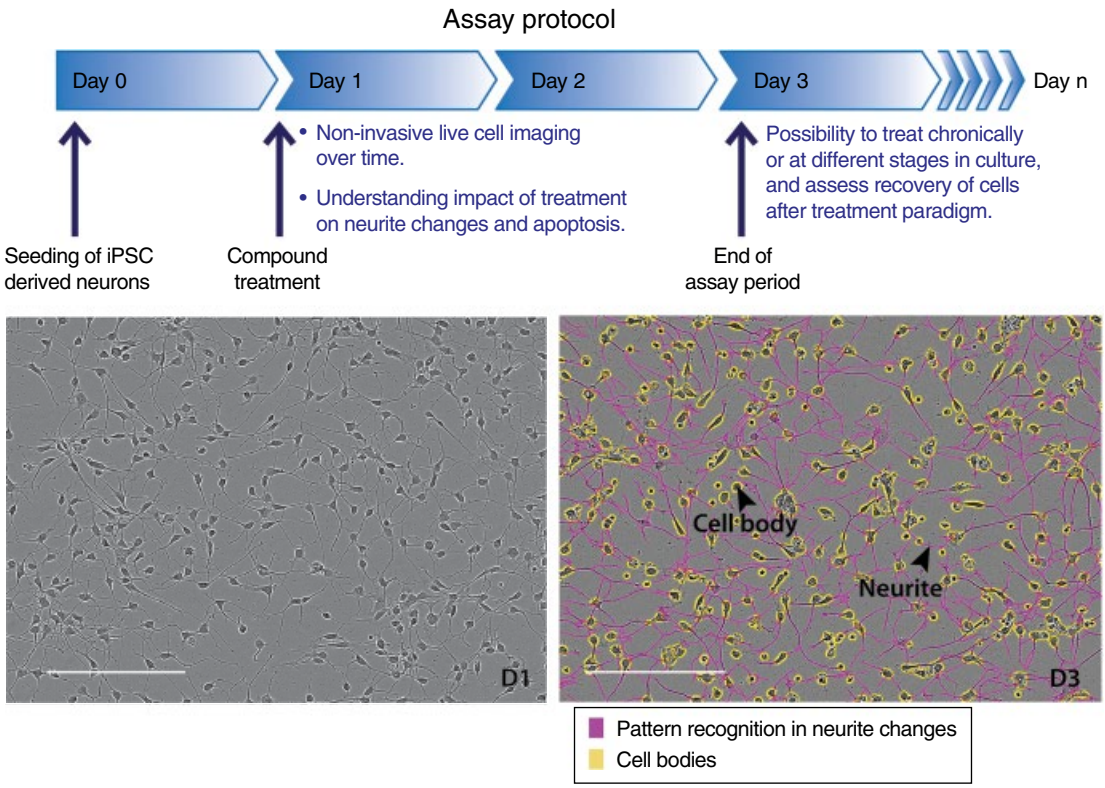

Plate 4 ***(Figure 9.3)*** *Flexible dosing and scanning scheme for non-invasive live cell imaging over time using iPSC derived neurons. Cell cultures are maintained in a CO_2 and temperature controlled setting. Changes in neurite dynamics are observed during the experimental timeline, including apoptosis by Caspase 3/7 measurement (not shown). Pattern recognition software enables analysis of neurite length and cell bodies within each well measured over a period of time. D1 indicates Day 1 when the cells are treated and D3 represents Day 3 as the end of assay period. Scale for images is 200 µm*

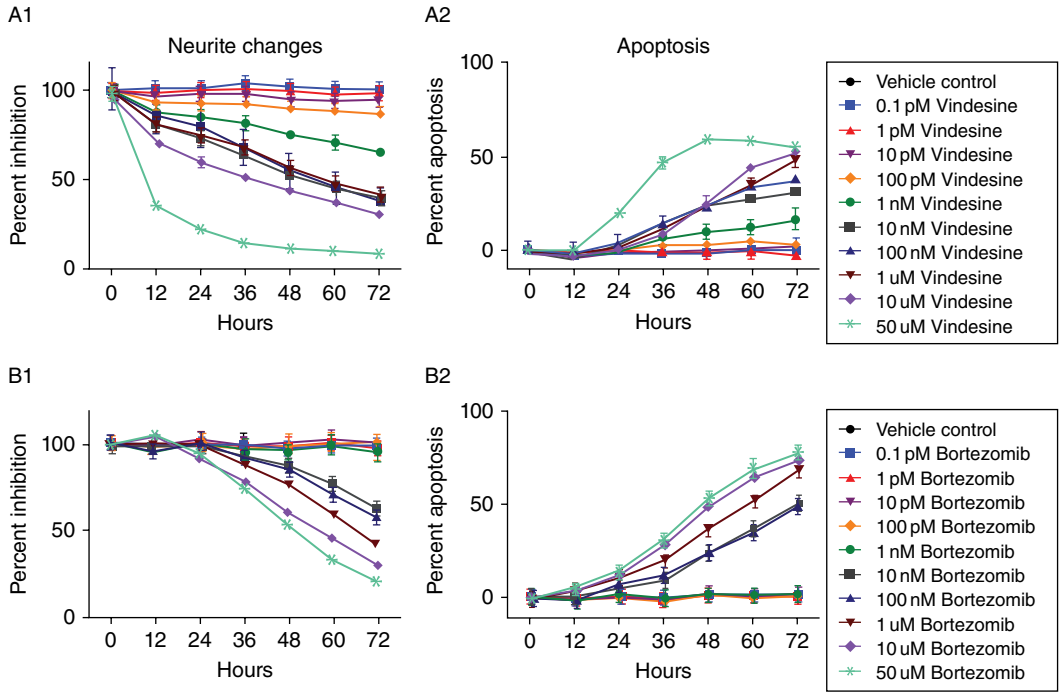

Plate 5 ***(Figure 9.4)*** *Differences in neurite changes and apoptosis upon treatment with varying doses of microtubule de-stabilizers or proteasome inhibitors on iPSC derived neurons over time. Live cell imaging over time and the capability to incorporate multi-parametric approaches in this assay enables the identification of molecules with different mechanism of action and may increase the sensitivity and neuro-specificity of the assay. Panel A. Microtubule de-stabilizer Vindesine induces neurite changes (A.i) prior to detection of apoptosis (A.ii). Panel B. Proteasome inhibitor Bortezomib induces concomitant toxicity in both parameters (B.i and B.ii)*

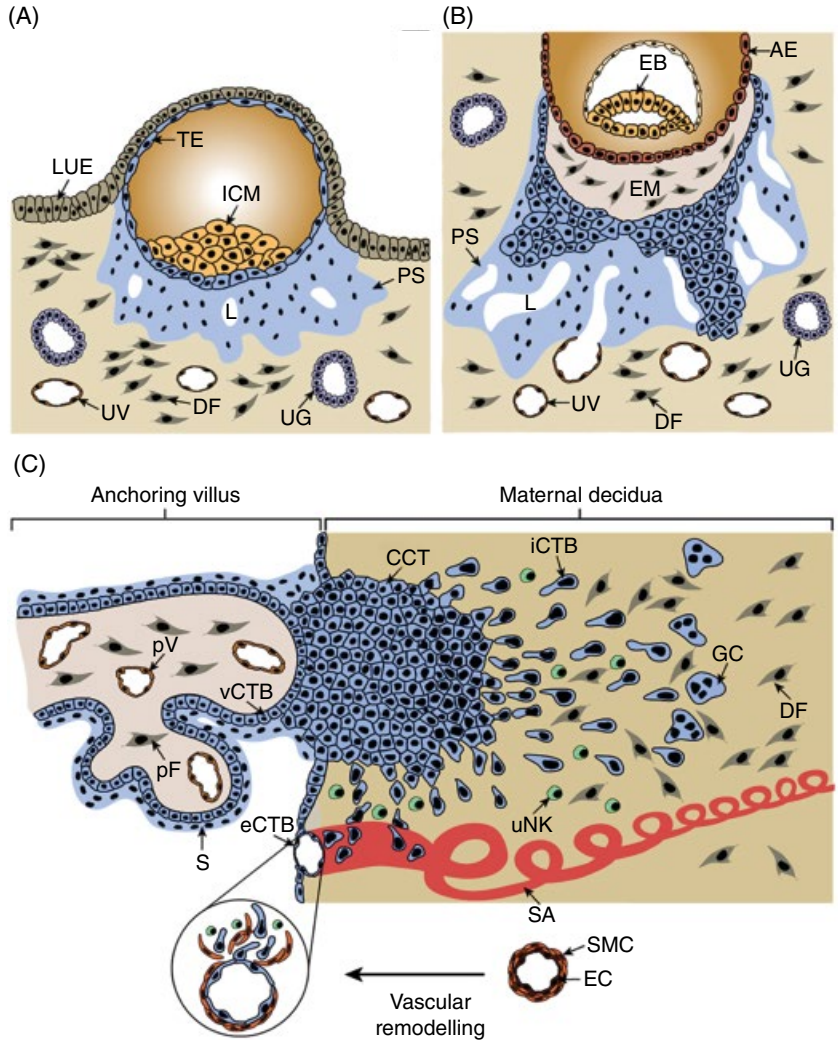

Plate 6 ***(Figure 10.1)*** *Cartoon illustrating some stages in human placental development (Knofler and Pollheimer 2013). (A) After implantation, the conceptus has sunk below the luminal epithelial cells (LUE) of the uterine wall, and proliferating cells of polar trophectoderm (TE) give rise to a primitive syncytium (PS) by cell fusion. This cellular structure appears to be able to invade into the decidualized endometrium and interact with decidual fibroblasts (DF). Non-cellular areas, called lacunae (L) soon inter-connect with uterine vessels (UV), fill with blood, and are the precursors of the intervillous space. (B) Soon after implantation, columns of proliferating cytotrophoblast (CTB) grow through the syncytium to form primary villi. AE, amnion; UG, uterine gland. (C) The architecture of placental villi and the maternal-fetal interface of the human placenta towards the end of the first trimester of pregnancy. Two kinds of villi are encountered: floating villi unattached to maternal endometrium (not shown) and an anchoring villus (shown) that attaches the fetal placenta to the uterine wall. All the villi are covered with a thin layer of STB (S) above villous cytotrophoblast (vCTB) that provides the exchange surface of the placenta. Note that the STB (S) is directly exposed to maternal blood at its apical surface. The core of the villus is comprised mainly of placental connective tissue (pF) and blood vessels (pV). Extravillous trophoblast forms as columns (CCT) at the tips of the anchoring villi and invade into the maternal decidual tissue (DF). A subpopulation of extravillous trophoblast (eCTB) penetrates maternal spiral arteries (SA) and replaces the resident smooth muscle cells (SMC) and endothelium (EC). Other interstitial types (iCTB) penetrate more deeply into the endometrium and encounter maternal NK cell (uNK). So-called giant cells, which are areas of syncytium, are also present in the endometrium, but their origin is not clear. They may arise through fusion of extravillous trophoblast or be remnants of primitive syncytium from the early invasion stages (James, et al. 2012; Knofler and Pollheimer 2013)*

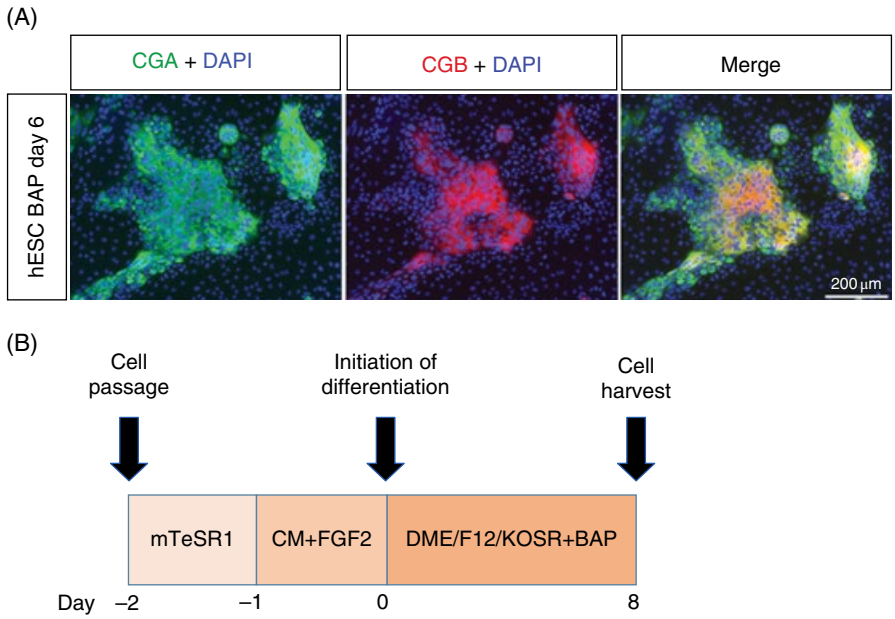

Plate 7 ***(Figure 10.2)*** *STB emerging within colonies of H1 ESC after six days of BAP treatment (A) and illustration of the ESC/BAP differentiation procedure (B). The region shown here has been stained by immunofluorescence localization for CGA and CGB, and by DAPI for nuclear material. The third panel in (A) shows the merged images. These regions of developing syncytium stain for antigens known to be expressed in placental STB. These include CGA and CGB (shown here). CGA generally becomes expressed earlier in the formation of STB than CGB*

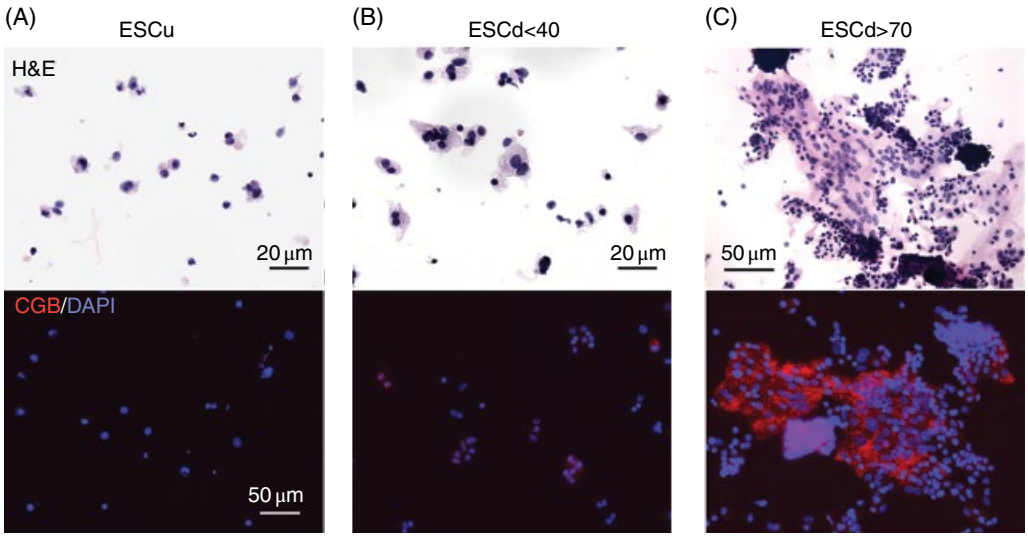

Plate 8 ***(Figure 10.3)*** *Images of three cell populations discussed in the text. (A) H1 embryonic stem cells (ESCu) stained by haemotoxilin/eosin (H & E, top) and stained by immunofluorescence localization of CGB and DAPI. The colonies of ESC were completely dissociated, and dispersed cells collected on a glass slide by using a Cytospin centrifuge procedure (www.thermoscientific.com/en/product/cytospin-4-cytocentrifuge.htmle). (B) Same as above, except the colonies had been differentiated to trophoblast by the BAP procedure (ESCd < 40) and fractionated by filtration through a sieve (40 μm mesh size). Note that some fragments of STB are present (top) and that a few clumps stain faintly for CGB (bottom). (C) Same as above, except showing cell fractions retained by a sieve with a mesh size of 70 μm (ESCd > 70). Note the presence of many nuclei in extensive cellular sheets (top), most of which stain strongly for CGB (bottom)*

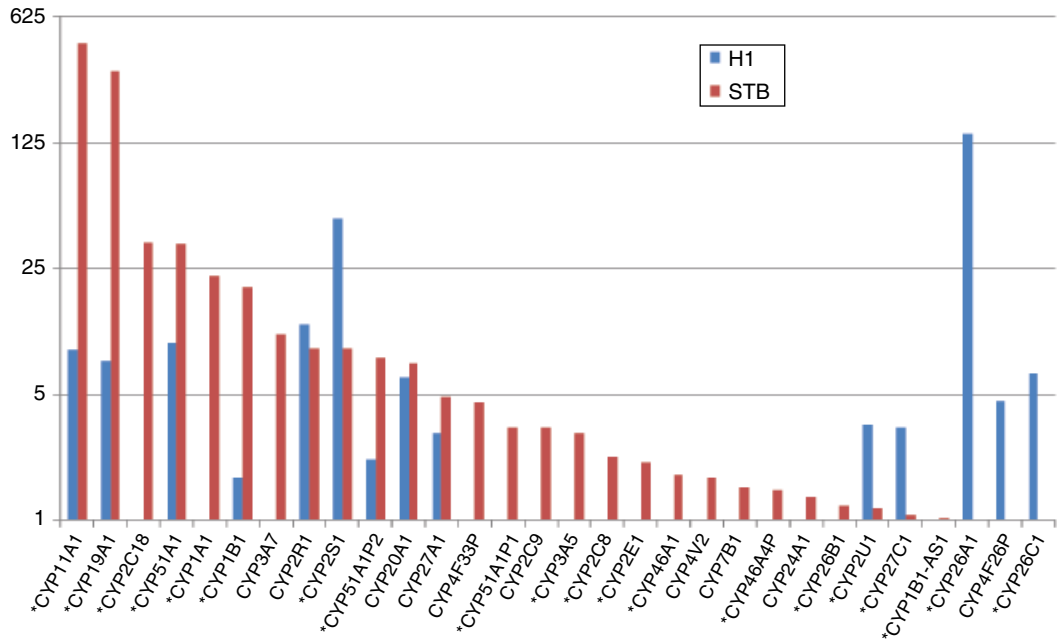

Plate 9 ***(Figure 10.4)*** *Relative expression of CYP genes in H1 ESC (ESCu, blue/dark gray) and STB (ESCd>70, red/light gray). Genes (on the abscissa) marked with an asterisk (*) were expressed differently by the two cell types (FDR<0.05). Data were obtained by RNAseq on cells at different passage numbers on three separate occasions. The differential expression analysis was performed by using Cufflinks. All values of<0.01 were recorded as 0.01 to simplify data presentation. Gene expression values (FPKM; fragments per kilobase of exon per million reads) are shown on the ordinate axis*

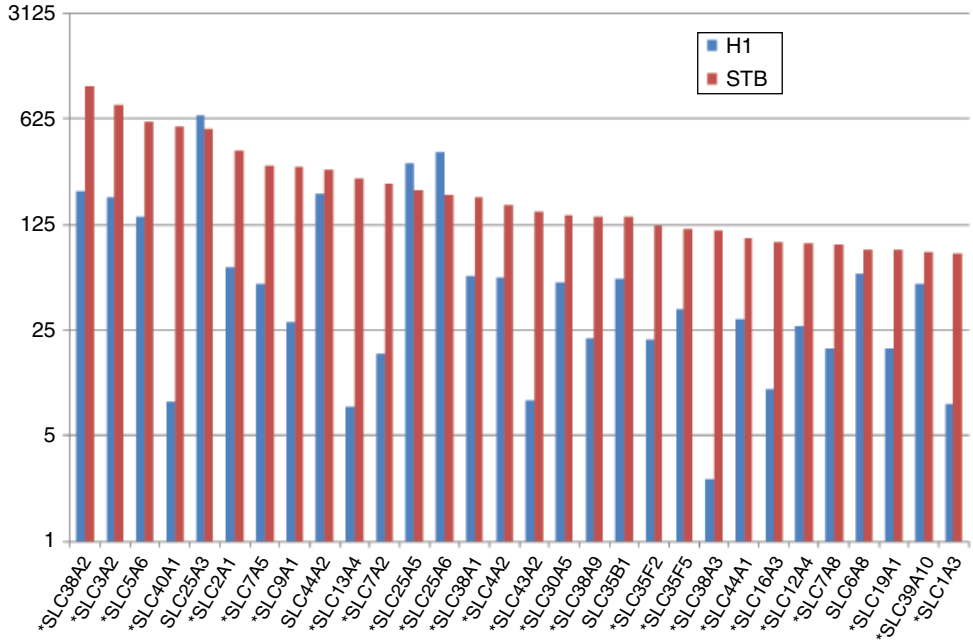

Plate 10 ***(Figure 10.5)*** *Relative expression of SLC genes in H1 ESC (blue/dark gray) and STB (red/light gray). Genes (on the abscissa) marked with an asterisk (*) were expressed differently by the two cell types (FDR<0.05). Presentation of data is described in Fig. 10.4. However, note the log scale for FPKM values on the ordinate*

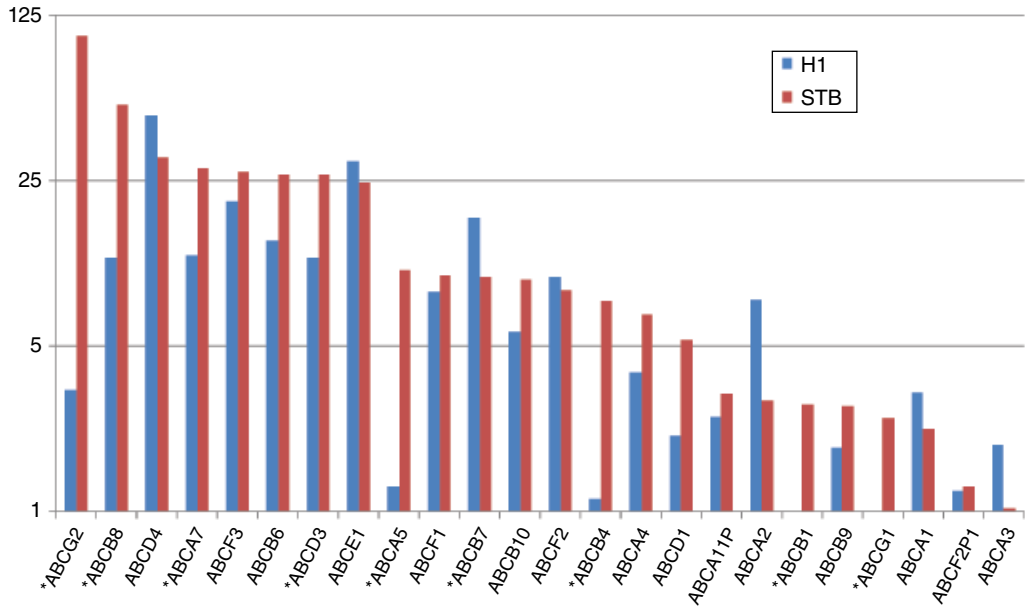

Plate 11 ***(Figure 10.6)*** *Relative expression of ABC genes in H1 ESC (blue/dark gray) and STB (red/light gray). Genes (on the abscissa) marked with an asterisk (*) were expressed differently by the two cell types (FDR < 0.05). Presentation of data is described in Fig. 10.4. However, note the log scale for FPKM values on the ordinate*

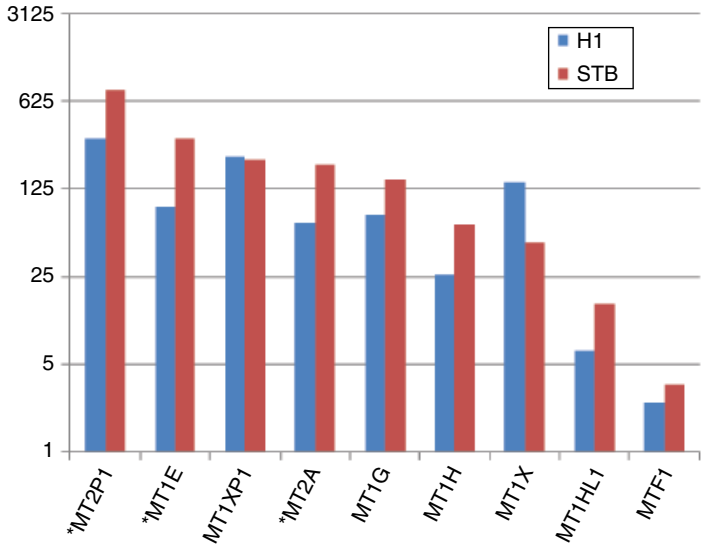

Plate 12 ***(Figure 10.7)*** *Relative expression of MT genes in H1 ESC (blue/dark gray) and STB (red/light gray). Genes (on the abscissa) marked with an asterisk (*) were expressed differently by the two cell types (FDR < 0.05). Presentation of data is described in Fig. 10.4. However, note the log scale for FPKM values on the ordinate*

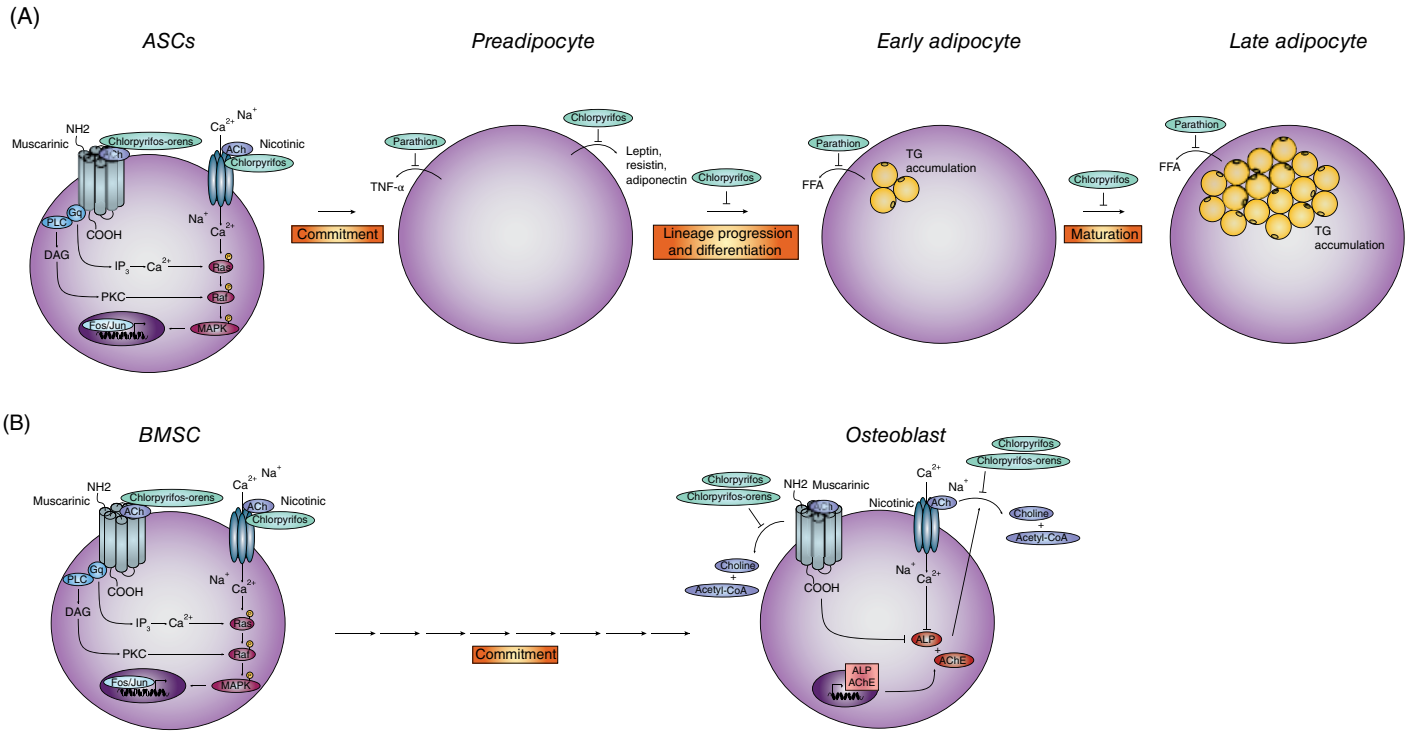

Plate 13 ***(Figure 11.1)*** *(A) The effects of organophosphate pesticides on ASC differentiation into adipocytes. (B) The effects of organophosphate pesticides on BMSC differentiation into osteoblasts*

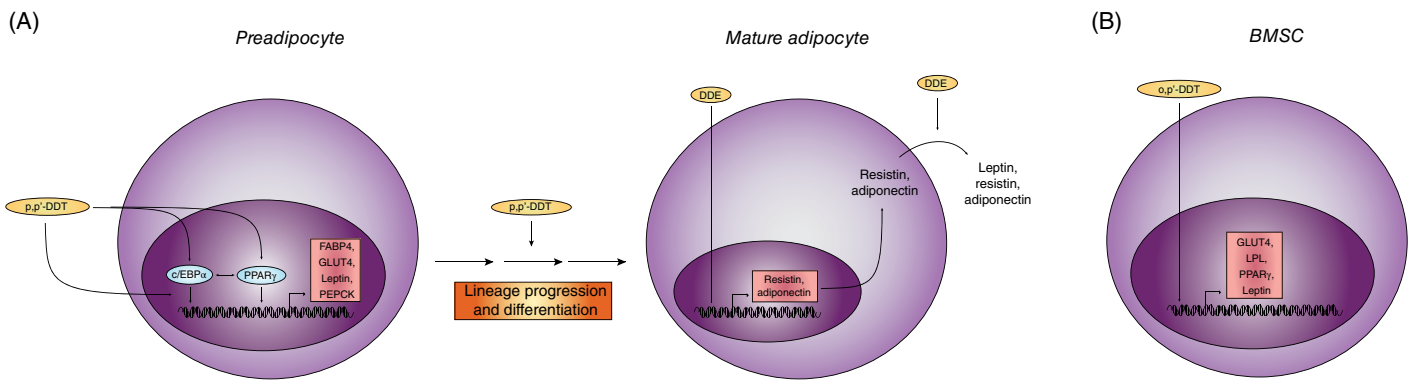

Plate 14 ***(Figure 11.2)*** *(A) The effects of DDT on preadipocyte differentiation into adipocytes. (B) The effects of DDT on BMSC differentiation*

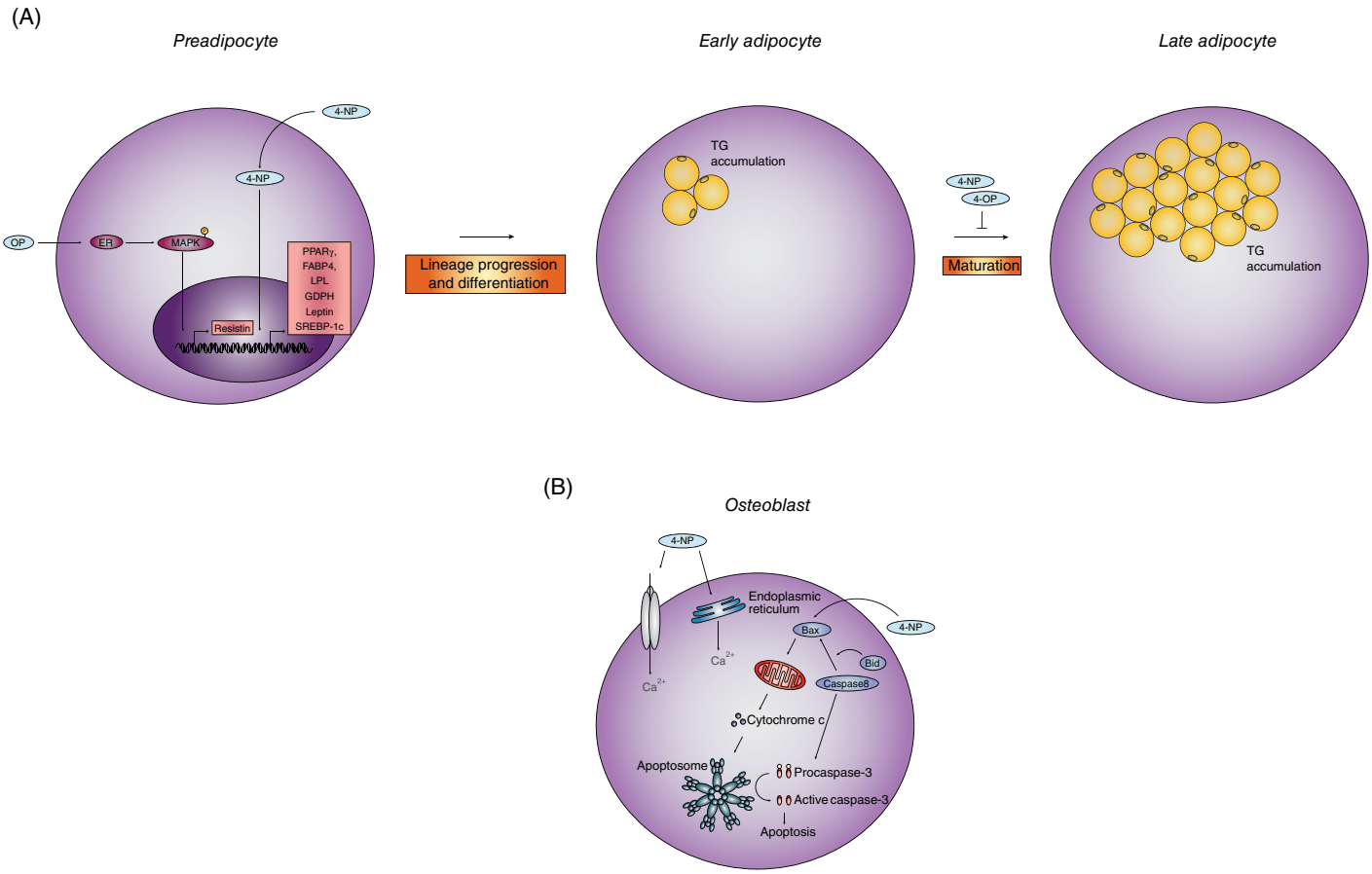

Plate 15 ***(Figure 11.3)*** *(A) The effects of alkylphenols on preadipocyte differentiation into adipocytes. (B) The effects of alkylphenols on induction of apoptosis in osteoblasts*

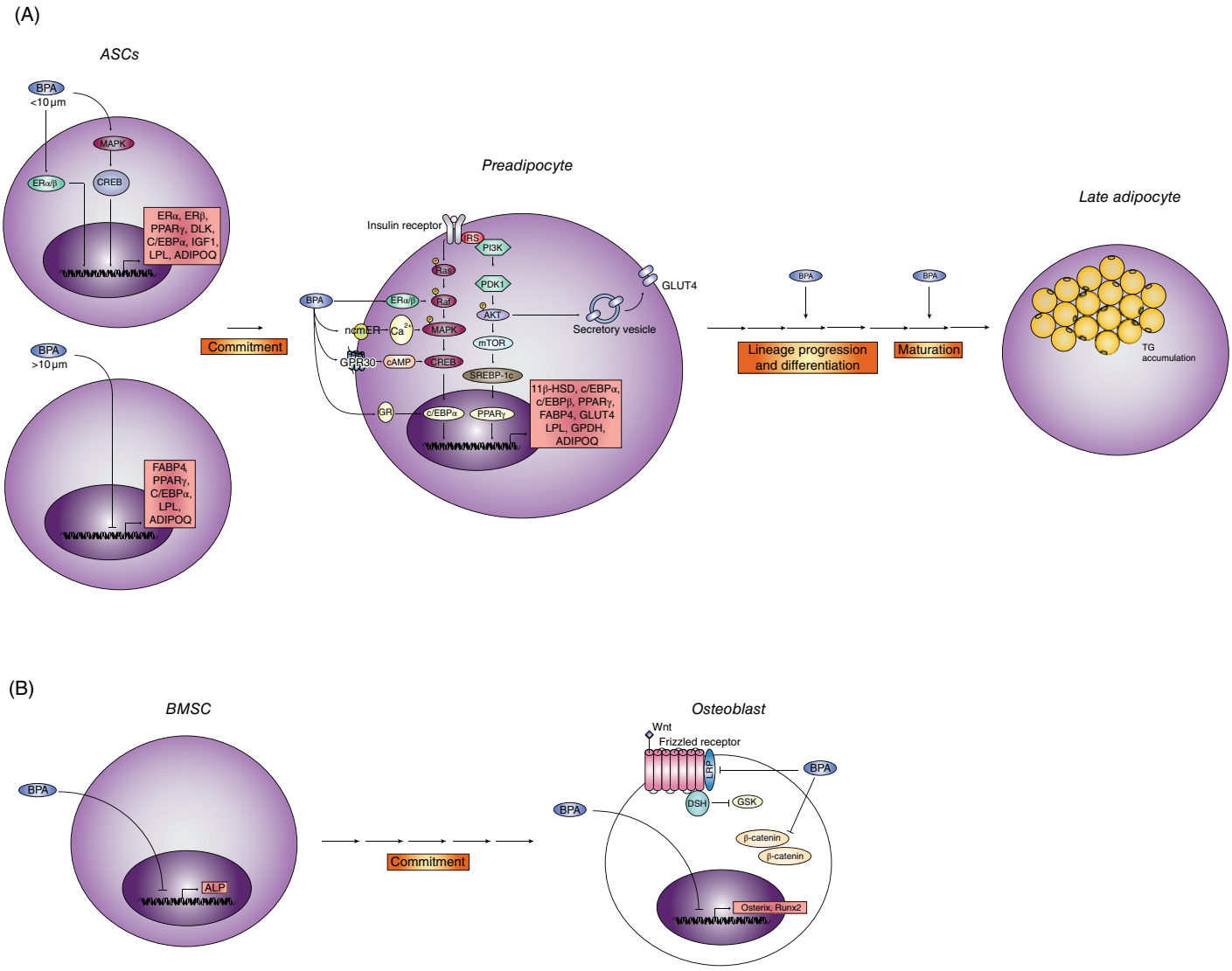

Plate 16 ***(Figure 11.4)*** *(A) The effects of BPA at <10 μM and >10 μM concentrations on ASC differentiation into adipocytes. (B) The effects of BPA on BMSC differentiation into osteoblasts*

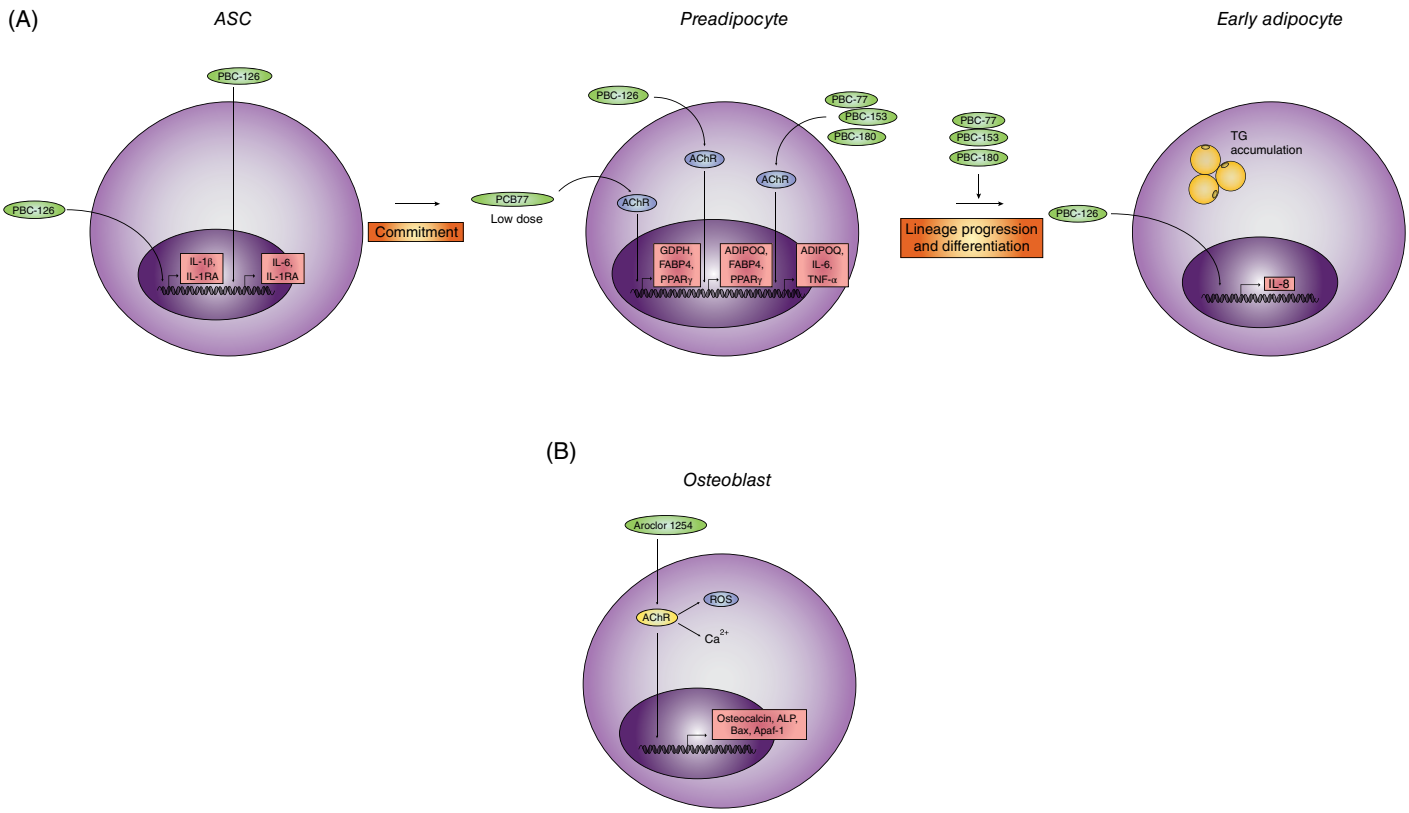

Plate 17 ***(Figure 11.5)*** *(A) The effects of PCBs on ASC differentiation into adipocytes. (B) The effects of PCBs on osteoblasts*

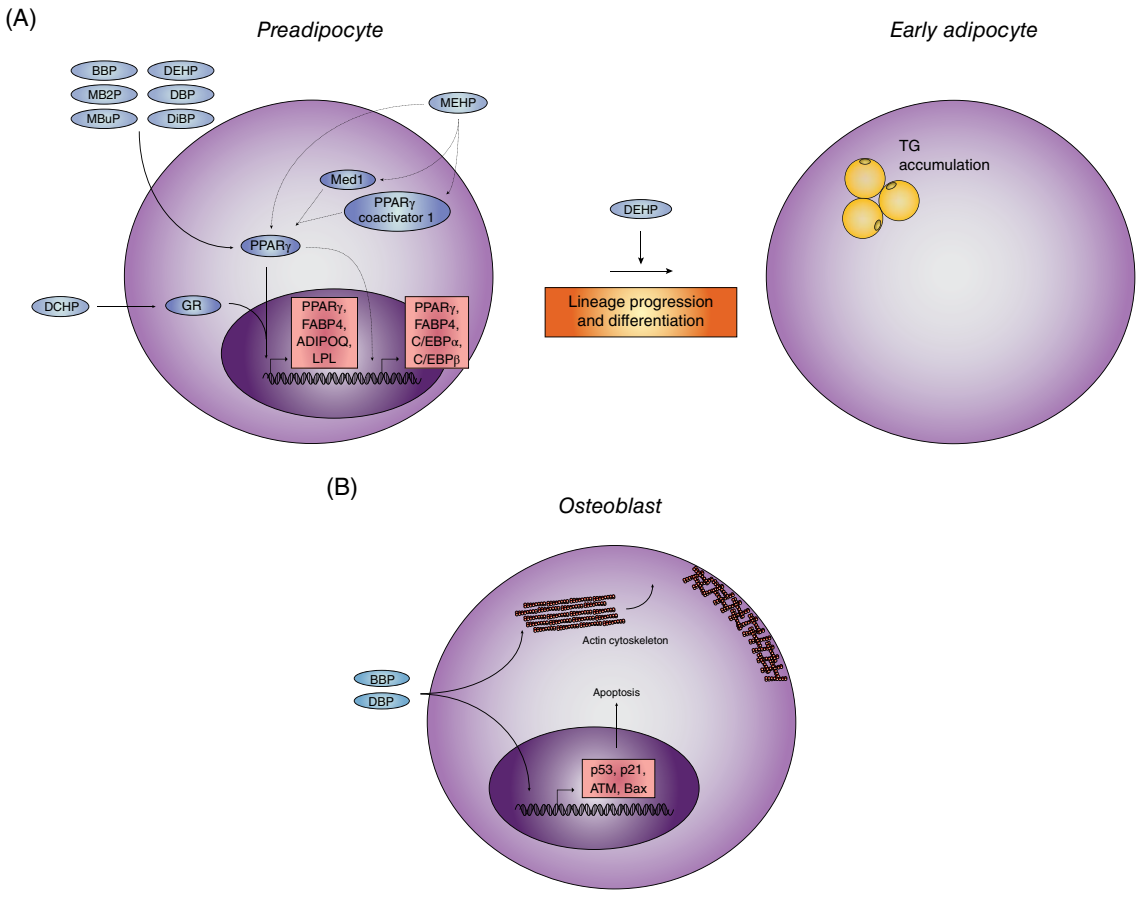

Plate 18 ***(Figure 11.6)*** *(A) The effects of phthalates on preadipocyte differentiation into adipocytes. (B) The effects of phthalates on osteoblasts*

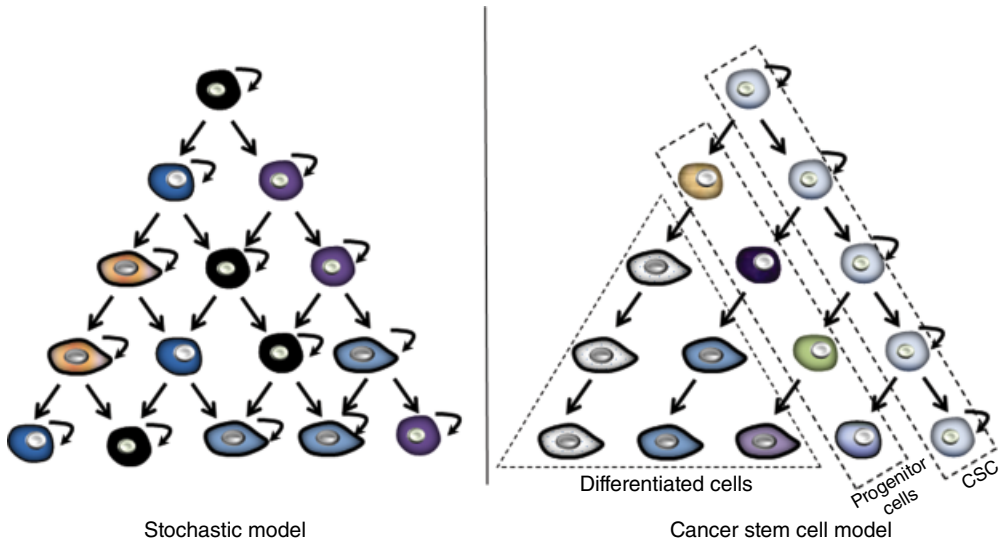

Plate 19 (Figure 20.1) Proposed models of cancer development and heterogeneity. *The stochastic model of cancer development advocates that all tumor cells have similar potential to divide and support growth of the tumor. These tumor cells are equipotent and can choose stochastically between self-renewal and differentiation. The cancer stem cell (CSCs) model of tumorigenesis proposes a hierarchical manner of cancer development and growth. According to this model, CSCs are the only cells with the potential to proliferate extensively and generate committed progenitor cells. These multiple progenitor cells can then further give rise to more differentiated cells, thus augmenting heterogeneity of the tumor mass*

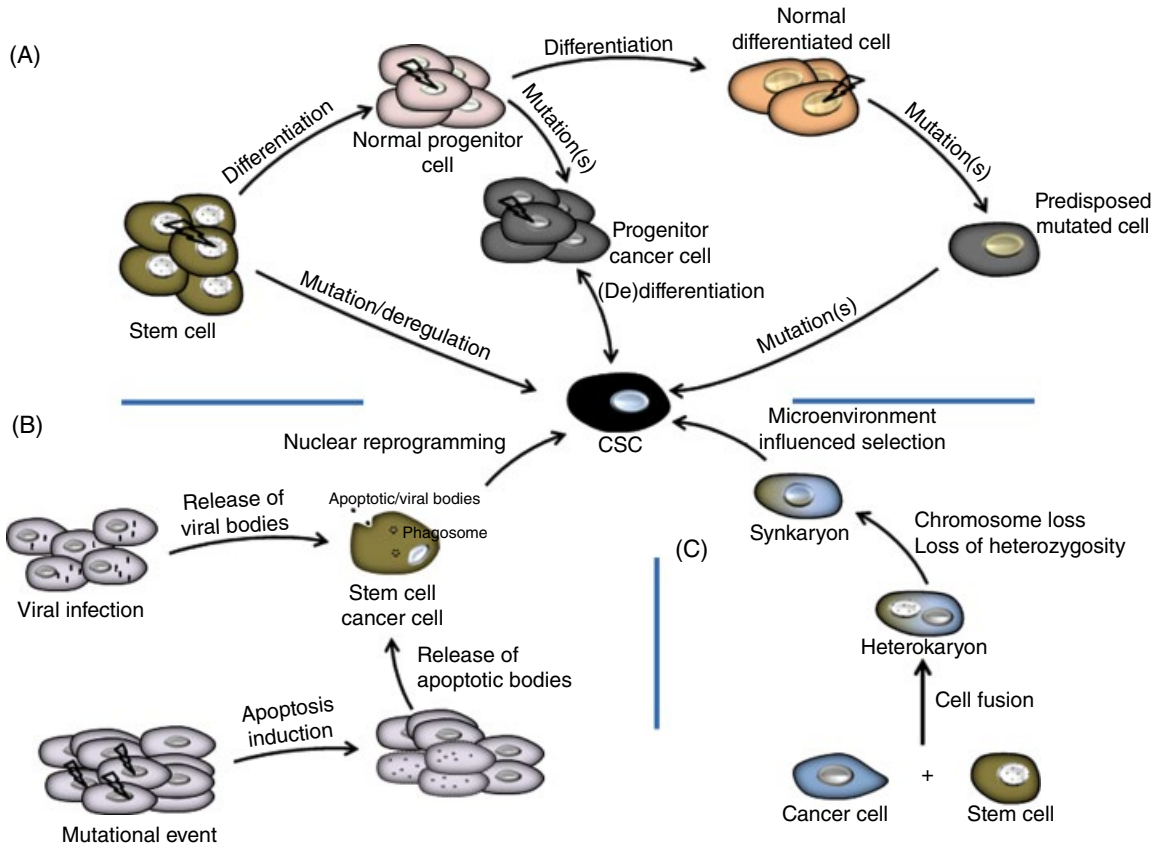

Plate 20 (Figure 20.2) Origin of cancer stem cells. *The presence of cancer stem cells (CSCs) has been verified in several tumors, and various hypotheses have been proposed to understand the formation of CSCs: (A) CSCs can arise as a result of mutation and/or generic abnormalities in a normal stem cell, or from the progenitor cancer cell generated upon mutations in normal progenitor cells which re-acquire self-renewal ability, and/or of from a normal cell which may acquire mutations predisposing it to form potential CSCs after further mutations; (B) apoptosis of somatic cells in response to stress may cause release of fragmented DNA. These fragmented DNA can be taken up by other stem/progenitor cells through endocytosis or phagocytosis causing nuclear reprogramming of the acceptor cell and formation of potential CSCs. Furthermore, due the presence of viral particles, viral oncogenes can also be taken up by stem cells potentially reprogramming it to generate CSCs; and (C) fusion of cancer cells with a normal stem cell can lead to the generation of heterokaryon (multinucleated cell) or synkaryon (mononucleated cell). Loss of heterozygosity in heterokaryons leads to the generation of synkaryons. These hybrid cell thus generated may possess self-renewal activity as well as properties of transformed cells, that is, properties of CSCs*

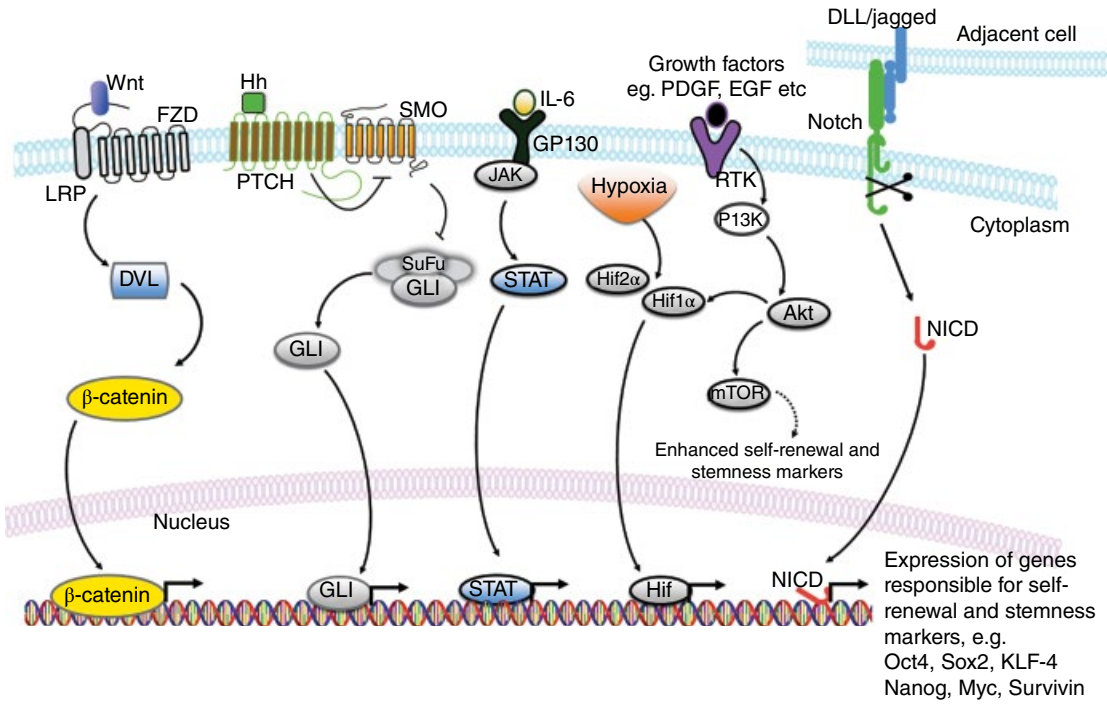

Plate 21 ***(Figure 20.3) Signaling pathways frequently utilized by cancer stem cells.*** *Transcription factors downstream of the represented signaling pathways have been associated in generation and maintenance of CSCs in different cancer types by upregulation of transcription factors such as Oct3/4, Sox2, Nanog, KLF-4, and so on, and stemness associated genes. These transcription factors further reinforce stemness and stem cell markers enhancing tumorigenecity and maintenance of CSC sub-population. DVL, Dishevelled homolog; EGF, Epidermal growth factor; FZD, Frizzed; GP130, membrane glycoprotein 130; Hh, Hedgehog; HIF, Hypoxia inducible factor; IL-6, interleukin-6; JAK, Janus kinase; mTOR, mammalian target of rapamycin; NICD, Notch intracellular domain; PDGF, platelet-derived growth factor; PI3K, Phosphoinositide 3-kinase; PTCH, protein Patched homolog; RTK, receptor tyrosine kinase; SMO, Smoothened; STAT, signal transducer and activator of transcription; SUFU, Suppressor of fused homolog; Wnt, Wingless-type*

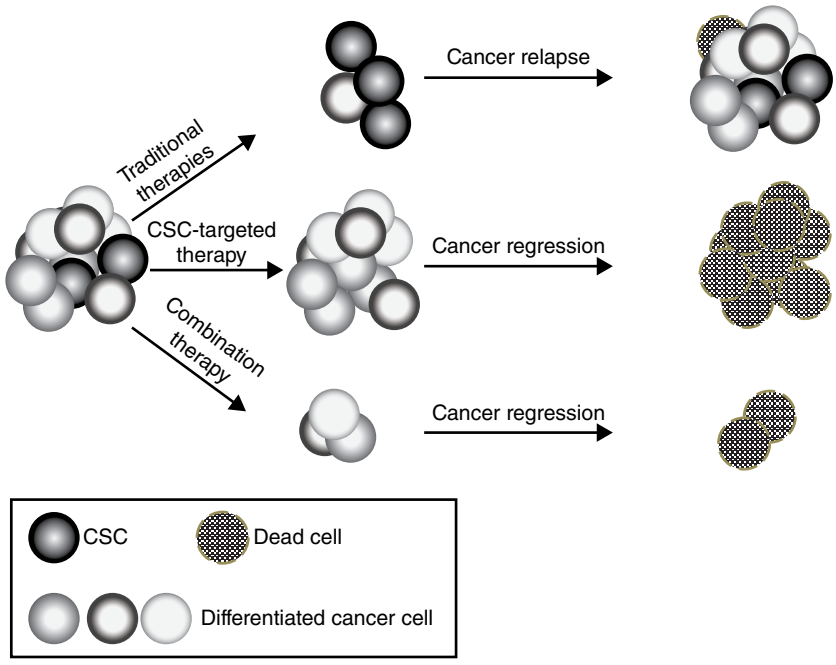

Plate 22 (Figure 20.4) A general overview of the concept of cancer stem cell-targeted therapy. *CSCs have been observed to be resistant to almost all of the current chemo- and radiation- therapies employed in the clinics. Thus, while an initial reduction in tumor burden is observed with these therapies, a relapse is almost always detected in these patients due to the surviving CSCs. Therefore, cancer therapies specifically targeting CSCs or a combination therapy targeting both the CSCs subpopulation and differentiated cells have been investigated to enhance therapeutic outcomes*

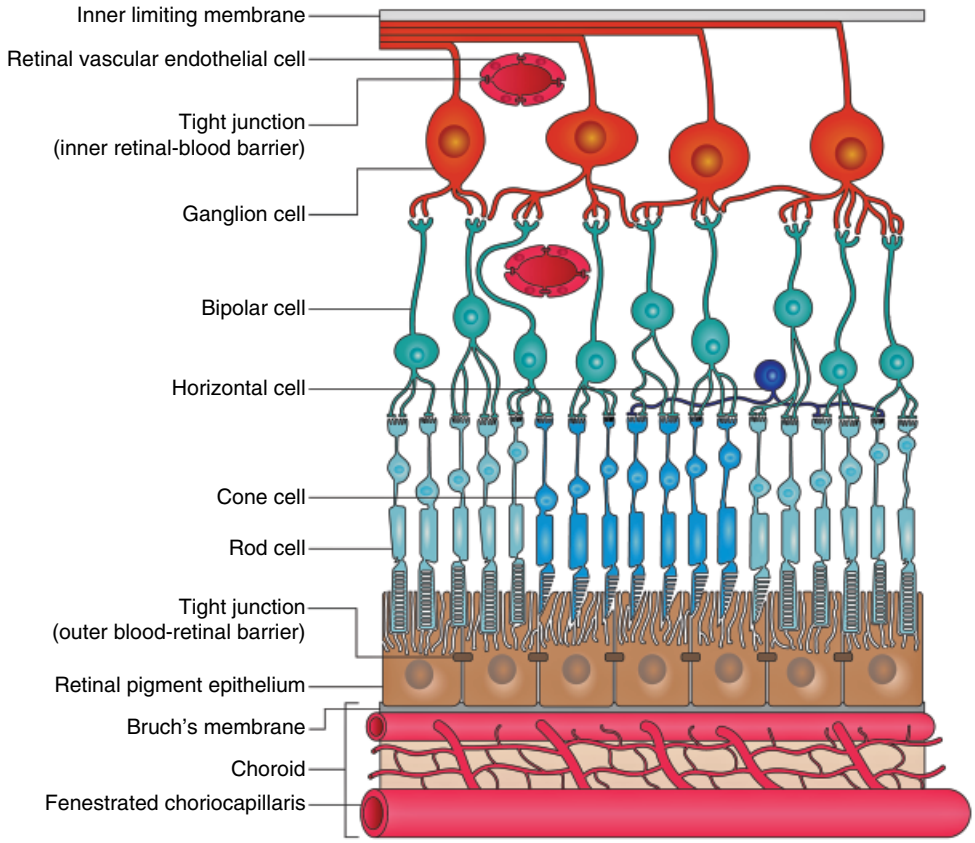

Plate 23 ***(Figure 22.1)*** *Diagram of a healthy human retina and outer blood-retinal barrier*

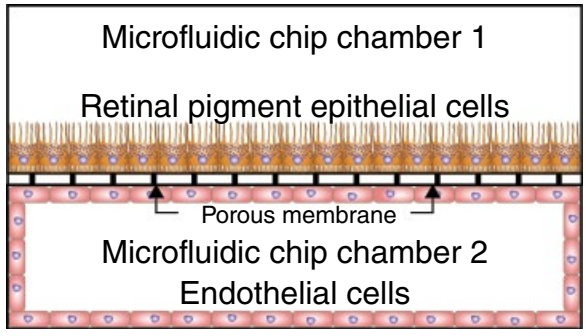

Plate 24 ***(Figure 22.2)*** *Example of an OBRB model in a microfluidic chip with RPE cells in chamber 1 separated by a porous membrane from chamber 2 with endothelial cells*

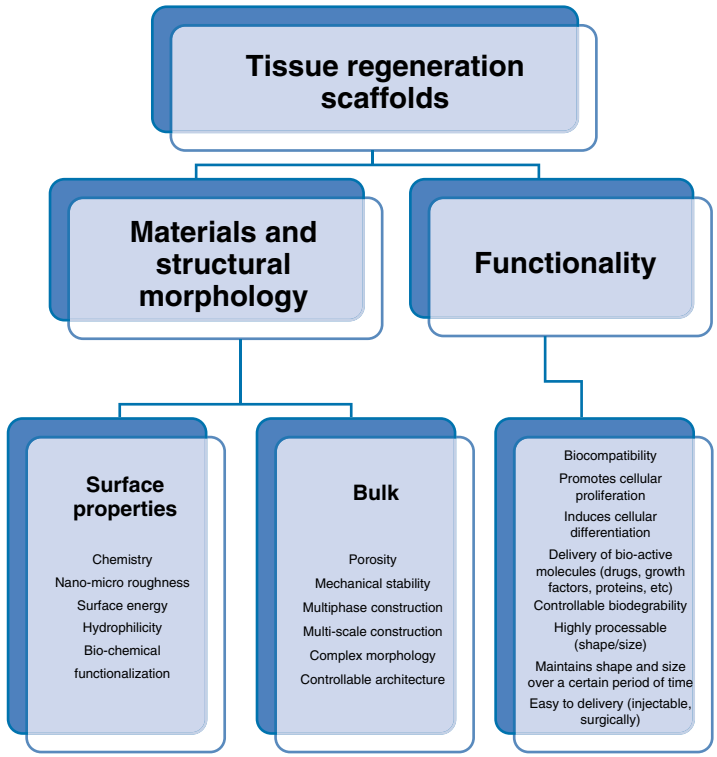

Plate 25 ***(Figure 23.1)*** *The complex set of requirements that a successful candidate for a tissue regeneration scaffold should include*

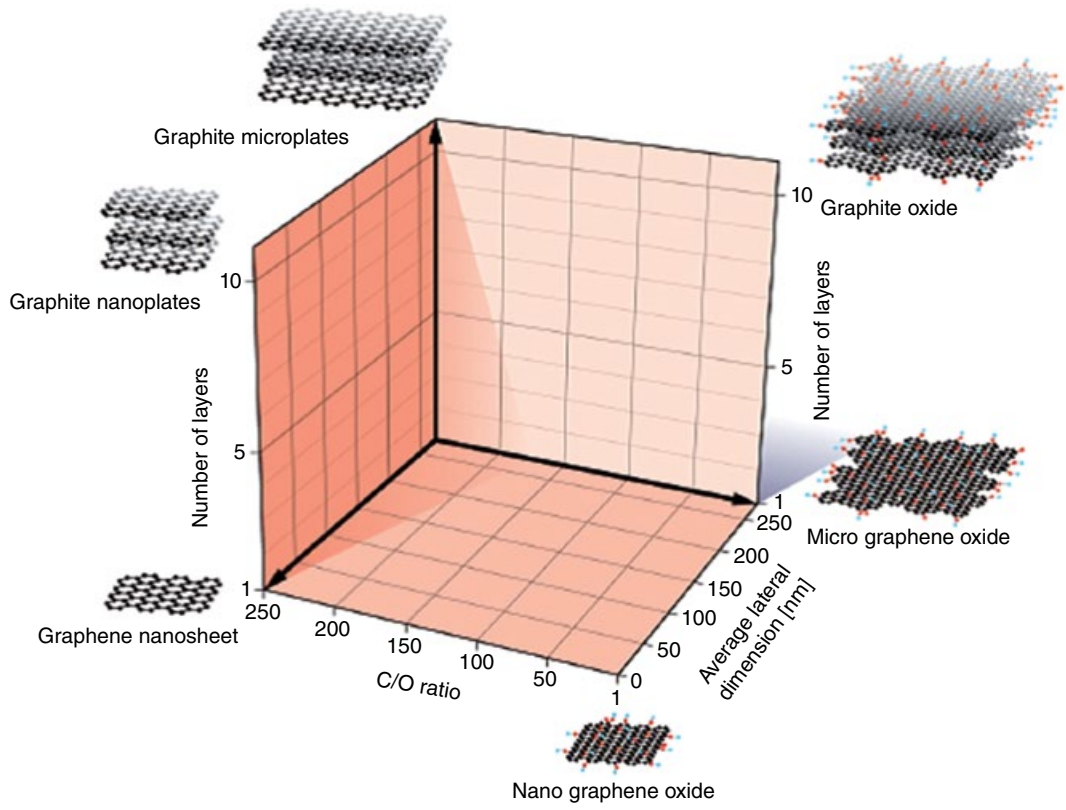

Plate 26 ***(Figure 23.2)*** *Schematic representing the various species of graphene structures. Adapted from Wick et al. [81], with permission of Wiley*

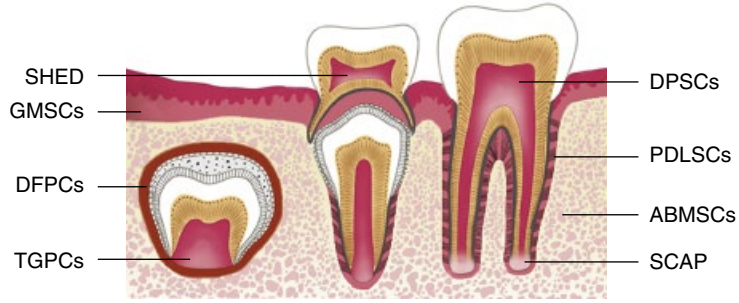

Plate 27 ***(Figure 24.1)*** *Schematic drawing illustrating sources of human dental tissue-derived MSCs. DPSCs: dental pulp stem cells; SHED: stem cells from exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; DFPCs: dental follicle progenitor cells; ABMSCs: alveolar bone-derived mesenchymal stem cells; SCAP: stem cells from the apical papilla; TGPCs: tooth germ progenitor cells; GMSCs: gingiva-derived MSCs*

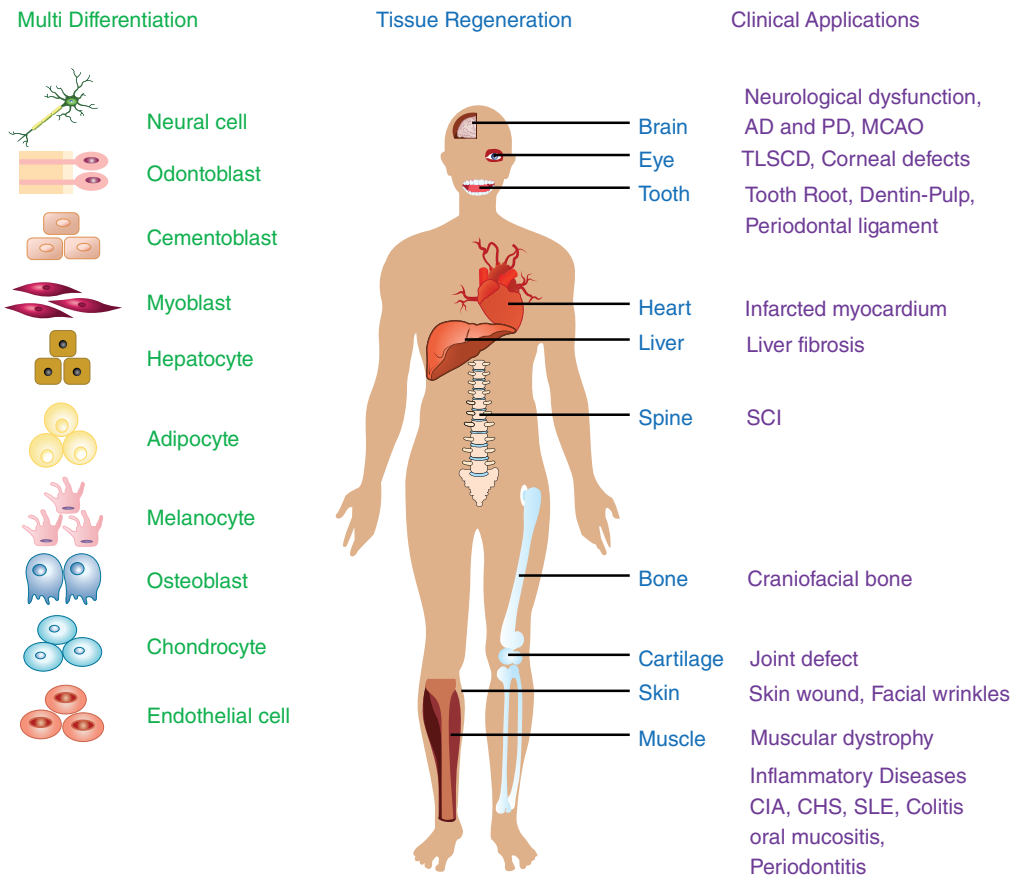

Plate 28 ***(Figure 24.2)*** *Multilineage differentiation capacity, tissue regeneration and potential clinical applications of human dental tissue-derived MSCs. AD: Alzheimer's dementia; PD: Parkinson's disease; MCAO: middle cerebral artery occlusion; TLSCD: total limbal stem cell deficiency; SCI: spinal cord injury; CIA: collagen-induced arthritis; CHS: contact hypersensitivity; SLE: systemic lupus erythematosus*

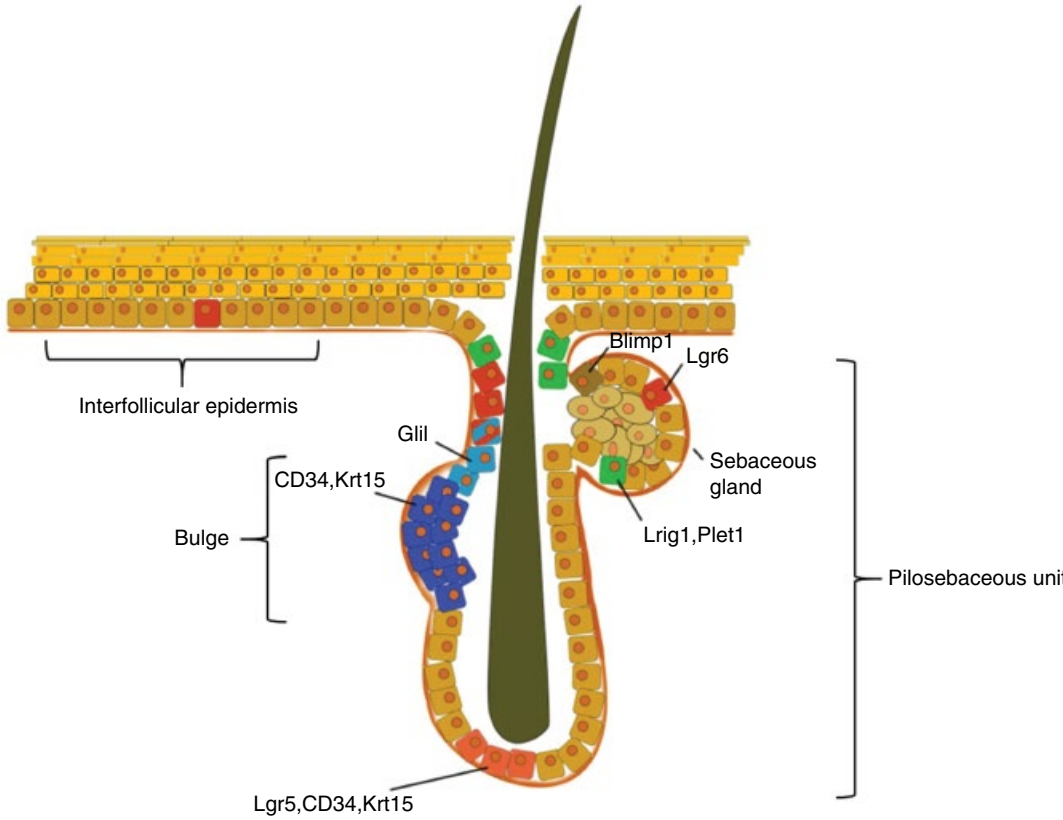

Plate 29 ***(Figure 25.1)*** *Distribution and the biological markers of skin stem cells*

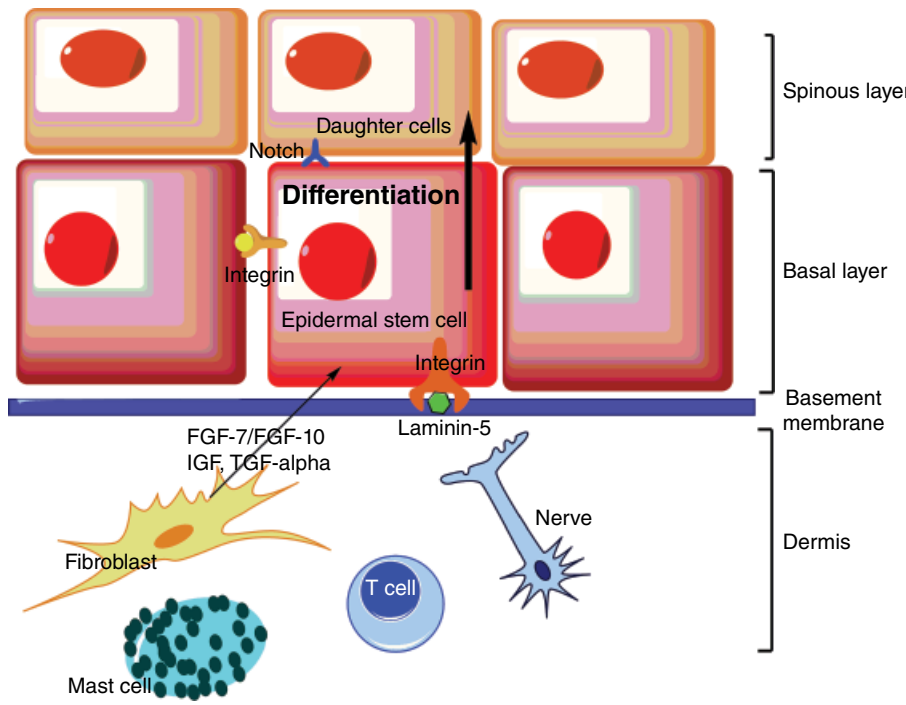

Plate 30 ***(Figure 25.2)*** *Model of skin stem cell niches*

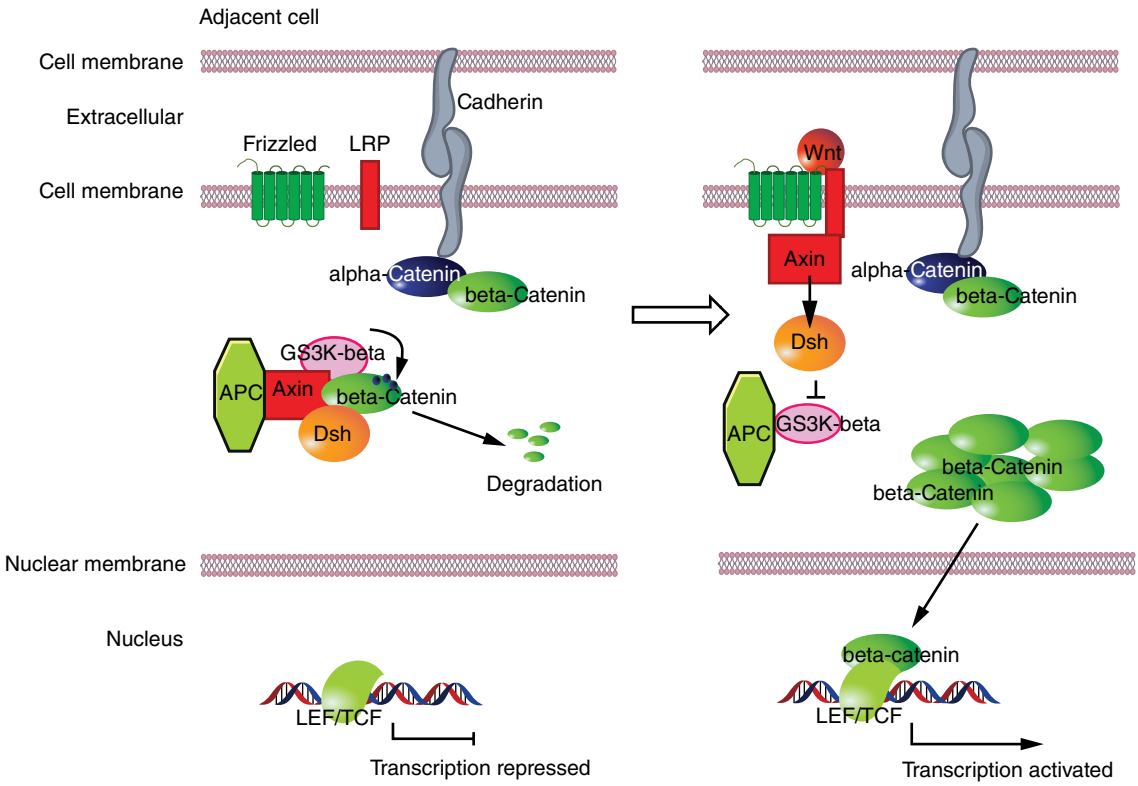

Plate 31 *(**Figure 25.3**) Wnt/β-catenin signaling pathway*

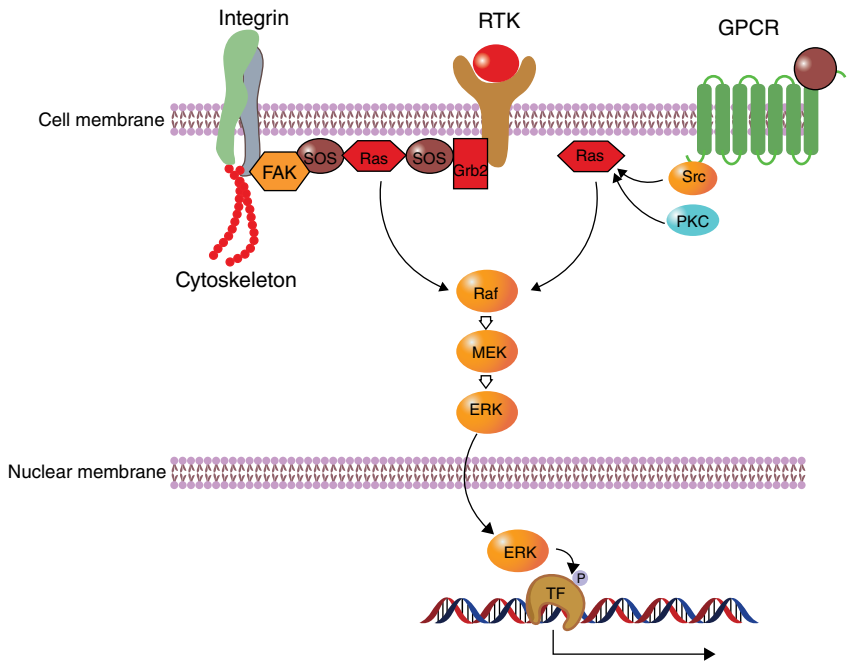

Plate 32 ***(Figure 25.4)*** *MAPK signaling pathway*

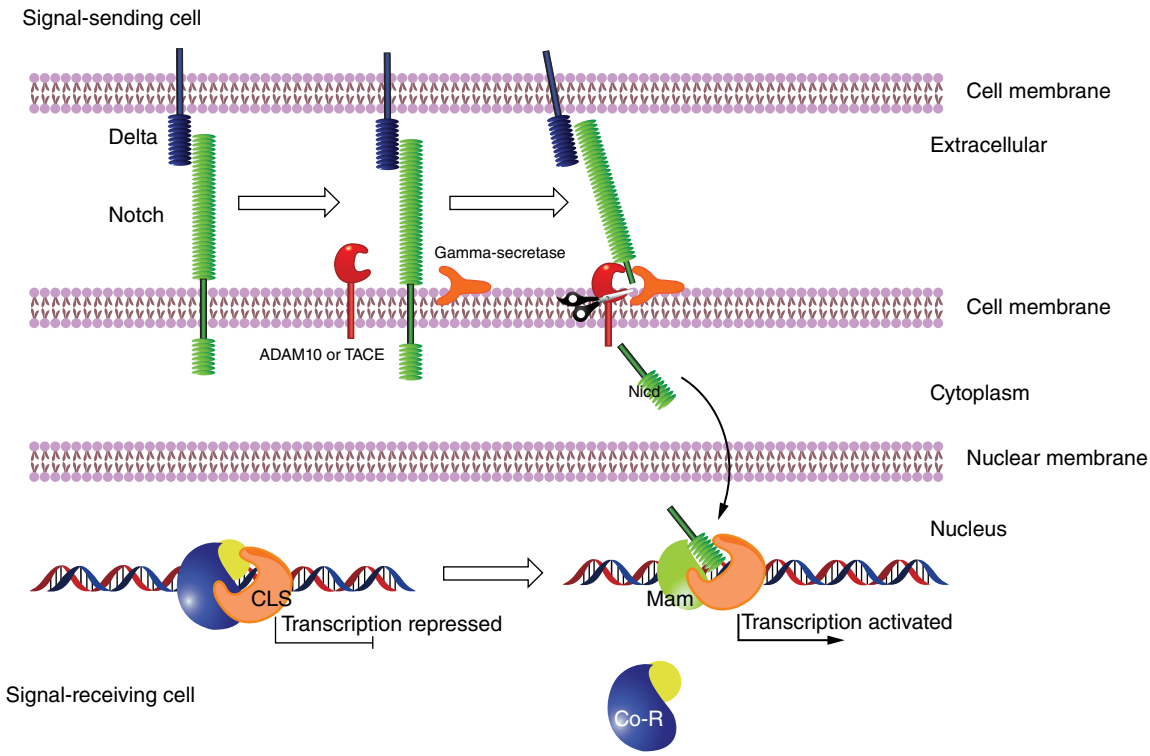

Plate 33 ***(Figure 25.5)*** *Notch signaling pathway*