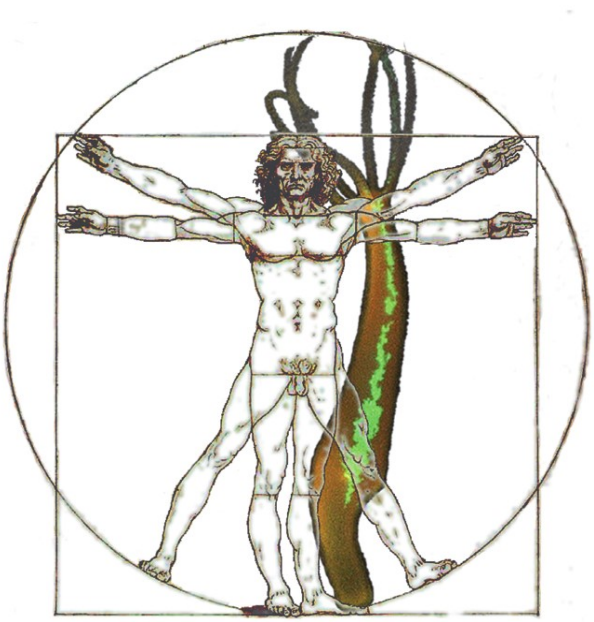
Thomas C.G. Bosch
Editor

Stem Cells

From Hydra to Man



Springer

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Editor
Prof. Dr. Thomas C. G. Bosch
Zoological Institute
Christian-Albrechts-University
Kiel, Germany

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Preface

There are two strategies to maintain a complex structure over a long period of time: Repair and preserve as good as you can; or re-build the identical complex continuously. To let our heritage go into a state of disrepair is not acceptable for most of us. Man-made buildings, therefore, need to be repaired and preserved continuously and lots of efforts are devoted to it. The Ise Shrine in the Mie Prefecture on the South East coast of Japan is maintained over hundreds of year by the alternative strategy: instead of repairing it continuously, the shrine is just rebuilt every 20 years. According to popular belief, in this way the site is purified and building materials renewed while preserving the original design from the third and fourth centuries A.D. The new shrines, however identical with the old ones, are not considered a replica of Ise, but are "Ise re-created." The recreation process reveals Shinto's understanding of nature which does not make monuments, but lives and dies, always renewed and reborn. Similar to house constructions, the adult body needs efforts, tools and methods to maintain its tissue and organs. That is what stem cells are used for in the adult body. Only stem cells have the ability to self-renew and to generate progeny capable of differentiating into one or more cell types. Numerous stem cell types are located in various depository sites and wait for demand, due to tissue maintenance in replacement of damaged or aged cells.

Much of our knowledge of stem cells has been inferred from studies of remarkable few species. The ability to manipulate stem cells in "model" organisms such as the mouse and a few other vertebrate species has driven our understanding of basic biology of stem cells. Data obtained suggest that a constellation of intrinsic and extrinsic cellular mechanisms regulates the balance of self-renewal and differentiation in all stem cells; the transcription factors Oct4 and Nanog, as well as the LIF-gp130-Stat3, BMP-TGF-b-Smad, MAPK-ERK and possibly the WNT signaling pathways, all have important roles in this process. Thus, an emerging theme is the implementation of the same signaling pathways in distinct stem cell systems. Questions about where stem cells are located, how they are maintained, what they can become, and the interchangeable nature of the stem and transit amplifying state are central topics of current research in vertebrate stem cell biology.

Organisms become models when they support sustainable opportunities with uncompromising experimental rigor and ease of use. The power and efficiency of studying model organisms, however, comes at a cost since a few species, obviously,

do not reflect nature's true diversity. Unfortunately, although all multicellular organisms seem to rely on stem cells, and although this seems to be a question of key importance for understanding the evolution of animal life, little is known about stem cells in early-branching taxa.

Therefore, welcome to ***Stem Cells: From* Hydra *to Man***. The title reflects an enormous growth in the knowledge of stem cells in various organisms over the past few decades. Large scale species comparisons at the genome and EST level have revealed that early-branching metazoans such as sponges and cnidarians share many if not most of their genes with the allegedly advanced vertebrates including man. The ancestor of all animals may thus have been much more complex than anticipated. Does that hold true also for the stem cells? Do all stem cells in the animal kingdom follow the same rules? Or are there real differences between stem cells in different organisms? The purpose of the book is to illustrate that here is more than human and mouse stem cells to learn from. The book presents the conceptual language and the nature of questions, as well as a summary of the advances in our understanding of stem cells from a comparative point of view that has resulted from the development of new technology and the development of novel model organisms over the past decade. As such this book is largely a horizon analysis of a frontier rather than a retrospective. It presents an integrative approach to animal stem cells and covers the major contributions, tools and trends in a newly emerging field: comparative stem cell biology.

We begin by considering stem cells in plants. Plants and animals may have evolved in quite different fashions. However, there is no doubt that plants and animals have evolved from a common eukaryotic ancestor, as for example indicated by the clear homology of genes that control the chromatin level of gene regulation. In plants, the shoot apical meristem can initiate organs and secondary meristems throughout the life of a plant. A few cells located in the central zone of the meristem act as pluripotent stem cells: They divide slowly, thereby displacing daughter cells outwards to the periphery where they eventually become incorporated into organ primordia and differentiate. **Jan U. Lohmann** reviews the latest information on plant stem cell regulation and how transcription factors and hormones control cell proliferation and maintenance of stemness, as well as how this ultimately leads to appropriate differentiation during plant development. We have included a chapter on plant stem cells in a book describing mostly stem cells in animals, since only by fully understanding the differences between plants and animals can we distinguish between those features of developmental pattern formation and cellular signaling that are necessary aspects of complex organisms, and those that are accidents of evolutionary history.

Although understanding stem cells in early-branching metazoan animals in general is a field in its infancy, we start to get interesting insights from one of the oldest multicellular organisms, the sponges (phylum Porifera). Based on her studies in the freshwater sponge *Ephydatia fluviatilis*, **Noriko Funayama** summarizes recent studies on the sponge stem cell system. She emphasizes that the stem cell system includes two types of pluripotent stem cells, archaeocytes and choanocytes and describes some of the molecular markers which recently came to light.

An important step during animal phylogeny was the invention of a tissue layer construction and nervous system within the phylum Cnidaria. Cnidarians, therefore, are an informative animal group separating from other metazoans prior to the origin of the bilaterian assemblage. Early work in hydrozoan cnidarians such as the freshwater polyp *Hydra* has shown that there are three distinct stem cell lineages. In Chapter 3, I summarize the current state of knowledge and the significant progress that has been made in recent years towards understanding the molecular control mechanisms involved in the *Hydra* stem cell system.

Macroscopic flatworms known as freshwater planarians (Platyhelminthes) possess an extraordinary stem cell system. A single cell type – pluripotent stem cells (neoblasts) – is responsible for cell renewal during growth, development, homeostasis and regeneration; neoblasts are also responsible for germ line formation. Since 1897 and appearance of a classic paper (H Randolph's "Observations and experiments on regeneration in planarians") these organisms, therefore, became attractive models to study stem cells. Similar to *Hydra*, research in Platyhelminthes has been revitalized by the recent application of the tools of molecular and cellar biology. Two papers in this volume describe that Plathelminthes are unique animals which we can utilize to understand fundamental mechanisms of stem cell systems. **Kiyokazu Agata** summarizes recent work on the planarian stem cell system at the cellular and molecular level. **Peter Ladurner** and colleagues introduce a rather novel model to study platyhelminth stem cell biology, the free-living flatworm *Macrostomum lignano*.

As an approximation of ancestral chordates, ascidians (Urochordata) can provide insight into the link between non-chordate deuterostomes and chordates, as well as the origination of vertebrates. Urochordates in fact are now considered the closest living relatives of vertebrates and because of their unique evolutionary position very valuable models. **Anthony W. De Tomaso** and colleagues summarize the renewing cell populations in adult ascidians and their role in regeneration and asexual proliferation. He shows that stem cells in ascidians are excellent models to study the biology of both embryonic and adult stem cells.

Can stem cells functionally contribute to human tissue repair? The answer is already known and is "yes". Three chapters in this volume show that both animal data and observations in humans indicate that stem cells may restore damaged organ function. **Makoto Asashima** and colleagues uses two types of undifferentiated cells, amphibian animal caps and mouse embryonic stem cells, to identify and characterize factors involved *in vitro* in maintenance of the undifferentiated state as well as the mechanisms regulating differentiation and control of organogenesisusing amphibian and stem cells.

Emerging evidence from stem cell research has strengthened the idea that stem cell fate is determined by a specialized environment, known as the stem cell niche. **Masatake Osawa** and colleagues summarizes studies in melanocyte stem cells (MSCs), which not only allowed the identification of individual stem cells in the niche, but also to obtain the molecular signature of individual MSCs in the niche. Since loss-of-function mutations in the genes responsible for MSC regulation in mice are readily identifiable by a prematuring hair greying phenotype, the MSC system appears to be an excellent model to study stem cell biology.

Embryonic stem cells are also a cornerstone of Singapore's National Biomedical Science Strategy. **William L. Rust** from Singapore's Institute of Medical Biology summarizes the state of the art of human embryonic stem cell research (hESC) aimed at generating tissues suitable for clinical use. Giving an outlook on the future directions of hESC research, the chapter provides a fascinating link between stem cells and their use in clinical settings.

Finally, there is emerging evidence that some blood cell cancers and solid tumors may contain a cancer cell hierarchy reminiscent of the normal tissue in which the malignancies first arose, with a cancer stem cell producing progeny with limited replication potential. The discovery of tumor cells that behave like stem cells suggests why cancer may be so hard to eradicate – and how new therapies might be targeted. Given the possible importance of cancer stem cells as therapeutic targets, **Holger Kalthoff** and **Ibrahim Alkatout** reviews recent advances in understanding the development of cancer and the role of cancer stem cells.

Taken together, the chapters in ***Stem Cells: From* Hydra *to Man*** reveal many common themes utilized in the maintenance and differentiation of stem cells of apparently disparate organs in animal and plant models. From an experimental point of view, each stem cell model system has its advantages and disadvantages. The book chapters show that although the molecular players controlling stem cell behavior are different in plants and animals, the overriding theme remains that signals and transcription factors are utilized to control pattern formation and differentiation from stem cell precursors. Across the animal kingdom there is a striking conservation of signalling and transcriptional mechanisms utilized in diverse stem cell differentiation processes. A comparative analysis of stem cells in diverse organisms, therefore, promises new insights into how stem cells act to construct and maintain tissues, and to reveals how the diverse stem cell systems may have evolved. Although applying genomic approaches to non-models is still challenging, the dichotomy between models and non-models is diminishing.

In conclusion, as the Ise shrine illustrates that death and constant renewal is the essence of nature, the work summarized in this book emphasizes the central role of stem cells as well as the mechanisms underlying their renewal capacity.

Thomas C. G. Bosch

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Contents

Contributors

Kiyokazu Agata
Department of Biophysics, Graduate School of Science,
Kyoto University Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502,
Japan, agata@mdb.biophys.kyoto-u.ac.jp

Ibrahim Alkatout
Department of General and Thoracic Surgery, Division of Molecular Oncology,
University Hospital Schleswig-Holstein, Campus Kiel, Christian-Albrechts-
University Kiel, Arnold-Heller-Str. 7, D-24105 Kiel, ialkatout@path.uni-kiel.de

Makoto Asashima
Department of Life Sciences (Biology), Graduate School
of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku,
Tokyo 153-8902, Japan, asashi@bio.c.u-tokyo.ac.jp

Thomas C. G. Bosch
Zoological Institute, Christian-Albrechts-University Kiel, Olshausenstrasse 40,
D-24098 Kiel, Germany, tbosch@zoologie.uni-kiel.de

Federico D. Brown
Institute for Stem Cell Biology and Regenerative Medicine, Stanford University,
Hopkins Marine Station, Pacific Grove, California 93950, USA

Katrien De Mulder
Institute of Zoology, University of Innsbruck, Technikerstrasse 25, A-6020
Innsbruck, Austria, Katrien.DeMulder@ugent.de

Anthony W. De Tomaso
Institute for Stem Cell Biology and Regenerative Medicine, Stanford University,
Hopkins Marine Station, Pacific Grove, California 93950, USA,
tdet@stanford.edu

Bernhard Egger
Institute of Zoology, University of Innsbruck, Technikerstrasse 25, A-6020
Innsbruck, Austria, Bernhard.Egger@uibk.ac.at

Noriko Funayama
Department of Biophysics, Graduate School of Science,
Kyoto University Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan,
funayama@mdb.biophys.kyoto-u.ac.jp

Kiyotaka Hasegawa
Cutaneous Biology Research Center, Massachusetts General Hospital,
MGH East, Building 149, 13th Street, Charlestown, Massachusetts 02129, USA

Holger Kalthoff and Ibrahim Alkatout
Department of General and Thoracic Surgery, Division of Molecular Oncology,
University Hospital Schleswig-Holstein, Campus Kiel, Christian-Albrechts-
University Kiel, Arnold-Heller-Str. 7, D-24105 Kiel, hkalthoff@email.uni-kiel.de

Georg Kuales
Institute of Zoology, University of Innsbruck, Technikerstrasse 25, A-6020
Innsbruck, Austria, Georg.Kuales@uibk.ac.at

Akira Kurisaki
Department of Life Sciences (Biology), Graduate School
of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku,
Tokyo 153-8902, Japan

Peter Ladurner
Institute of Zoology, University of Innsbruck, Technikerstrasse 25, A-6020
Innsbruck, Austria, peter.ladurner@uibk.ac.at

Jan U. Lohmann
Max Planck Institute for Developmental Biology, Spemannstrasse 37-39,
D-72076 Tübingen, Germany, jlohmann@tuebingen.mpg.de

Mariko Moriyama
Cutaneous Biology Research Center, Massachusetts General Hospital, MGH East,
Building 149, 13th Street, Charlestown, Massachusetts 02129, USA

Tatsuo Muchiue
Department of Life Sciences (Biology), Graduate School
of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku,
Tokyo 153-8902, Japan

Shin-Ichi Nishikawa
Center for Developmental Biology
2-2-3 Minatojima, Minami-machi, Chuo-ku, Kobe 650-0047, Japan

Masatake Osawa
Cutaneous Biology Research Center, Massachusetts General Hospital, MGH East,
Building 149, 13th Street, Charlestown, Massachusetts 02129, USA, masatake.
osawa@cbrc2.mgh.harvard.edu

Daniela Pfister
Institute of Zoology, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria, Daniela.Pfister@uibk.ac.at

William Rust
Institute of Medical Biology, A STAR (Agency for Science, Technology, and Research), 8A Biomedical Grove, #06-06 Immunos, Singapore 138648. wllmrust@yahoo.com.sg

Willi Salvenmoser
Institute of Zoology, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria, Willi.Salvenmoser@uibk.ac.at

Lukas Schärer
Institute of Zoology, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria, Lukas.Scharer@uibk.ac.at

Stefano Tiozzo
Institute of Medical Biology A*STAR (Agency for Science, Technology, and Research), 8A Biomedical Grove, #06-06 Immunos, Singapore 138648. wllmrust@yahoo.com.sg

Chapter 1
Plant Stem Cells: Divide et Impera

Jan U. Lohmann

Abstract Stem cells are an essential and defining feature of multicellular organisms. Since multicellularity arose independently in the plant and animal kingdom, it follows that also the stem cell concept has evolved independently in the two linages. Nevertheless, there are striking similarities in the way plants and animals organize their stem cell pools, suggesting that there might have been strong evolutionary constraints that shaped the path for the development of stem cell systems. This is illustrated by the fact that in both worlds, stem cell promoting signals are usually absent from the stem cells themselves, but rather found in neighboring cells, which provide an inductive cellular environment, also called niche. While there are perplexing similarities with regards to the overall stem cell concept, there are also profound differences. Arguably the most important disparity lies in the capacity of plants to maintain totipotent stem cells throughout their entire lives and that these cells are directly responsible for giving rise to the vast majority of the cellular mass of the adult plant body. Another fundamental difference between plants and animals lies in the dramatic developmental plasticity of plant cells, which allows them to take on multiple fates during their life. Therefore, plants have evolved a complex regulatory network, which allows for a precise control of stem cell proliferation and cell fate specification.

Keywords ant stem cell, meristem, WUSCHEL, CLAVATA, Plant hormore

1.1 Introduction

When a baby is born we marvel at its perfection: Eyes, hands, feet – all body structures are elaborated with stunning precision and in miniature size. Thus, while it will take years for the baby to grow, mature, and learn to become an adult, the basic body

Center for Organismal Studies, University of Heidelberg,
im Neuenheimer Feld 230, D-69120 Heidelberg, Germany.
E-mail: jlohmann@tuebingen.mpg.de

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plan is already laid down at birth. This so-called embryonic mode of development is not only limited to humans, but is in fact employed by most animals. In many species, immature offspring are even capable of life in the absence of further parental input.

While seedlings of plants can also survive without their parents, their mode of development differs dramatically from that of animals. Despite the fact that their embryos remotely resemble the adult structure, organs of embryonic origin do not contribute substantially to the adult plant. Instead, proliferative tissues called meristems, which harbor stem cells, continuously generate cells during the adult life phase to support growth and development of the plant (Fig. 1.1). Because plant cells are surrounded by a rigid, yet permeable, cell wall, they are unable to move within the organism by migration. Rather, cells are displaced passively by tissue streaming, which is driven by both cell proliferation and cell expansion. Cell proliferation is limited to meristems and to those cells that have recently been displaced from them. Most visible plant growth, however, is due to cell elongation and enlargement in differentiating organs and tissues. Thus, the body structure of a plant is elaborated and refined over the entire lifespan of the individual and is subject to constant change until senescence. This mode of development, generally referred to as postembryonic, allows plants not only to form new organs of various kind more or less continuously over their entire lifecycle, but also to regenerate

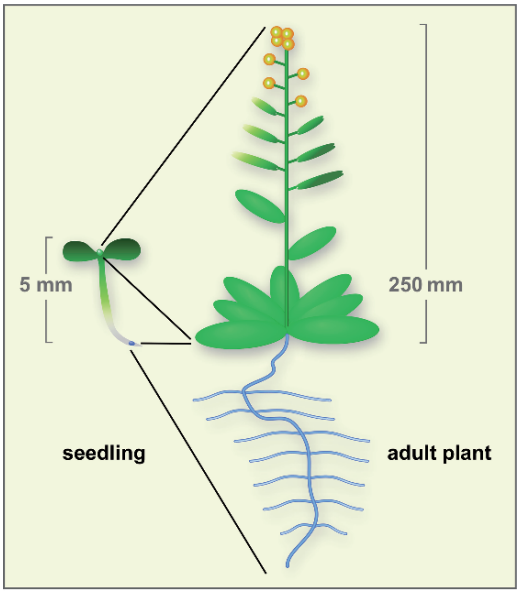

Fig. 1.1 Contribution of apical meristems to the plant body. The entire shoot system is derived from cells of the shoot apical meristem, while the root is derived from the root apical meristem. The contribution of embryonic tissues to the adult plant is marginal. Adapted by permission from Macmillan: Nature, Weigel and Jürgens, copyright 2002

lost structures with ease. A major challenge for plant development arises from their sessile lifestyle. While most animals can avoid harsh environmental conditions by seeking shelter or by migration, plants have to cope with diurnal as well as seasonal oscillations *in situ*. The adaptation to changes in light intensity, or to stresses such as heat, cold, or drought, not only require the plant to implement mechanisms to protect itself, but also to integrate these cues with a continuously active developmental program. As a result, plants have evolved a complex regulatory network to efficiently control the balance between stem cell proliferation and differentiation that functions over time frames lasting as long as thousands of years (e.g., for some tree species).

In this chapter I describe the structure and origin of the stem cell harboring tissue of the shoot, summarize what is known about the regulatory mechanisms underlying shoot stem cell homeostasis, and discuss some of the remarkable properties of plant stem cell regulation.

1.1.1 Structure and Function of the Shoot Apical Meristem

During embryogenesis two populations of totipotent stem cells are initiated that are dedicated to producing the shoot and the root, respectively (reviewed in Jürgens, 2001). These stem cell pools are embedded into specialized tissues called meristems. Despite the fact that the root system is of equal importance to the shoot and much is known about the root meristem, here I will focus on the stem cells of the shoot for the sake of simplicity.

The shoot apical meristem (SAM) is the source of all above ground tissues of a plant. It is one of the first structures to be initiated during embryogenesis (Fig. 1.2) and molecular markers for the SAM become expressed in a localized fashion by the 16-cell stage (Mayer et al., 1998). The presumptive SAM remains small and inactive throughout embryogenesis and acquires its full function during germination, when the embryo emerges from the seed coat (Jürgens et al., 1995; Laux and Jürgens, 1997; Jürgens, 2003).

In angiosperms, or flowering plants, the SAM of an adult plant is a dome shaped structure with a diameter of about 250 µm that contains a few hundred undifferentiated and dividing cells (Fig. 1.3). In contrast to cells in mature plant organs, which are large and vacuolated, meristematic cells are small and rich in cytoplasm. Despite the fact that cellular morphology is remarkably uniform across the meristem, not all cells in the meristem are stem cells – this fate is restricted to about 30 cells in the upper center of the meristematic dome. Cells that are born in the meristem are displaced to the periphery where they differentiate to form new organs, such as leaves and flowers.

Not unlike the germ layers in animals, the body of dicotyledonous plants is build from three clonally distinct tissue layers, termed L1, L2 and L3 (Fig. 1.3). L1 and L2 together form the superficial tunica and are both limited to a single layer of cells. The L3 layer in turn makes up the internal corpus. These layers are established

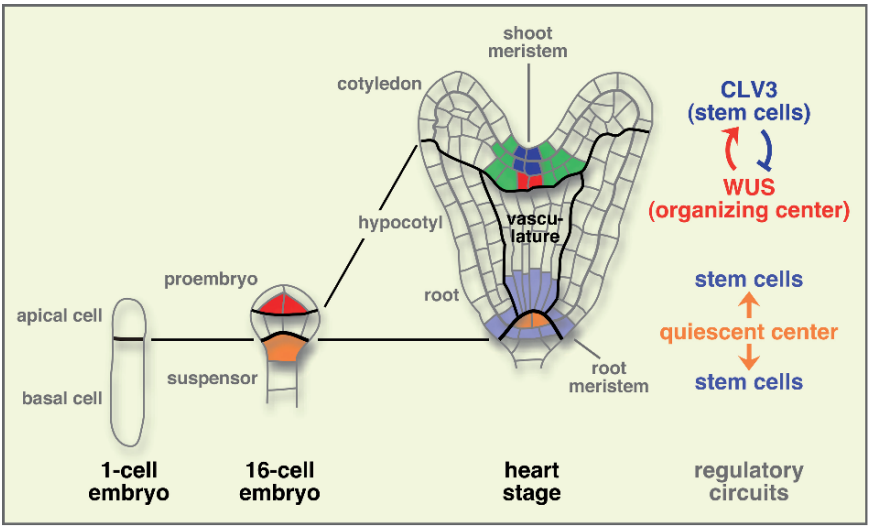

Fig. 1.2 Embryonic origin and molecular organization of apical meristems. The first marker for the identity of the shoot apical meristem is *WUSCHEL*, which is expressed at the 16 cell embryo and remains confined to a restricted domain throughout embryogenesis. Adapted by permission from Macmillan: Nature, Weigel and Jürgens, copyright 2002

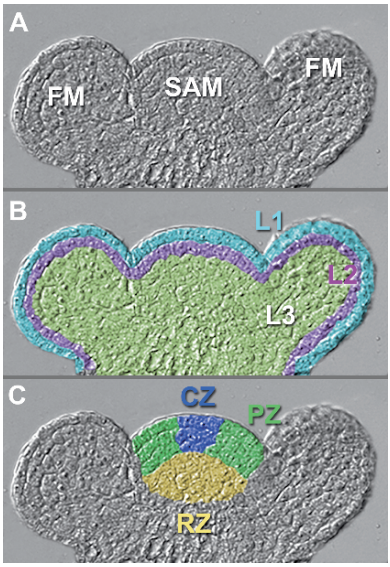

Fig. 1.3 Organization of the shoot apical meristem (SAM) during the reproductive phase. Longitudinal section through the center of the meristem are shown. (A) From the central SAM, which is home to the totipotent stem cells, floral meristems (FM) are formed, which will differentiate into mature flowers. (B) Tissue layer organization as observed at the SAM. Layers L1 and L2 form the superficial tunica, while L3 makes up the internal corpus. The layers remain clonally distinct by anticlinal cell divisions in L1 and L2. (C) Domain structure of the SAM. Totipotent and slowly dividing stem cells make up the central zone (CZ), while the cells of the peripheral zone (PZ), remain undifferentiated and divide more rapidly. The rib zone (RB) contains the stem cell inducing cells of the organizing center, which coincides with *WUSCHEL* expression

early during embryogenesis and remain separated over the entire course of plant development by anticlinal cell divisions in the L1 and L2 layers, while L3 cells divide in all planes. This layered organization is well reflected in the structure of the SAM and requires that cell proliferation and differentiation are coordinated by extensive cell-cell communication.

The organization into distinct tissue layers requires the presence of stem cells in all three layers and, consequently, cell behavior in the SAM is also highly regulated at the level of meristematic domains that include cells of different clonal origin (Fig. 1.3). About 20 to 30 slowly dividing and self-renewing totipotent stem cells make up the central zone, which spans the L1, L2 and L3 in the center of the meristem dome. Analysis of mericlinal chimeras, which can be induced by X-ray mutagenesis or transposon activation in living plant embryos of various species, has shown that the majority of cells in an adult plant are descendents of these stem cells. In *Arabidopsis thaliana*, for example, the cells of the entire embryonic meristem give rise to the first six leaves, while the remainder of the cell mass is clonally related to the stem cells (Irish, 1991). This was a remarkable finding, not only because it showed the important contribution of the true stem cells, but also because it highlighted the extent of cell proliferation in the meristem outside the stem cell domain. Indeed, adjacent to the central zone that harbors stem cells is a domain marked by rapid cell proliferation, which has been termed the peripheral zone (Grandjean et al., 2004; Reddy et al., 2004; Traas and Bohn-Courseau, 2005; Vernoux et al., 2000). Stem cell daughters that are themselves displaced from the central zone into the peripheral zone, loose stem cell identity and start to divide rapidly before they are incorporated into forming organs and subsequently differentiate. Thus, cells of the peripheral zone are similar to the so-called transit-amplifying cells of animal stem cell systems. Another feature of plant stem cells that bears striking similarity to animal stem cell systems is that they acquire their identity through signals emanating from neighboring cells. In the SAM these cells are embedded in the deeper layers of the meristem, known as the rib-zone. Only a small group of about 20–30 cells spanning three cell files below the central zone has stem cell inducing capacity. This group of cells is known as the organizing center (Mayer et al., 1998). Interestingly, while the organization of stem cell induction by neighboring cells is equivalent to the niche concept well established in animals, there are striking differences in how the stem cell niche is organized in the SAM. The most obvious difference is that not all stem cells have direct contact with the cells of the organizing center. Stem cells of the L1 and the cells in the lowest file of the organizing center are separated by six cell diameters and stem cells of the L1 and L2 are never in contact with the organizing center. This effectively rules out that molecules displayed at the cell surface constitute a stem cell inductive signal. Another major difference of the SAM stem cell system is the continuous turnover of the organizing center. Because the cells of the organizing center divide at very low rates, they are regularly displaced by cells leaving the stem cell domain during the growth of the plant. Thus, a plant cell is able to switch from stem cell fate to organizing fate and finally to a differentiation program purely based on its position in the organism. Elegant cell ablation studies in the root meristem have confirmed

this experimentally (van den Berg et al., 1995; van den Berg et al., 1997) and support the notion that plant cells have an extraordinary developmental plasticity. Recent studies using laser ablations of entire meristematic domains have shown that the SAM is able to recover from the loss of all stem cells within a relatively short period of time (Reinhardt et al., 2003a). Moreover, these experiments revealed that even the ablation of the organizing center in addition to the stem cells can be overcome by regulatory processes. Thus, the SAM is a highly plastic stem cell niche that provides an extremely robust environment for the long-term maintenance of totipotent stem cells.

1.1.2 Stem Cell Regulation at the SAM

In the following section I will discuss the molecular mechanisms of stem cell control in the SAM. Again, for the sake of clarity, I will not give a comprehensive overview, but rather focus on the key players.

Since the SAM is laid down in the embryo, genetic screens aimed at finding regulators of plant embryogenesis were very successful in identifying mutations affecting meristem regulation (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998; Long et al., 1996; Mayer et al., 1998). However, only a handful of genes with mutant phenotypes resulting in severe meristematic defects were isolated, which essentially fall into two classes: mutants in which the SAM is arrested and non-functional on the one hand, and those in which the SAM is enlarged and marked by overproliferation of cells on the other hand.

The first gene that was cloned from the former class was *SHOOTMERISTEM-LESS (STM)*, which codes for a homeodomain transcription factor of the plant specific KNOX class (Long et al., 1996). *STM* shows a SAM specific expression with *STM* transcripts excluded from cells that are part of an organ primordium. Thus, the absence of *STM* RNA is one of the earliest markers for differentiation of meristematic cells that morphologically are indistinguishable from other cells in the SAM. Consistent with this, inactivation of *STM* causes premature cell differentiation within the SAM and, consequently, a breakdown of stem cell maintenance. Thus, while *STM* plays a central role for SAM function by allowing cells to proliferate, it does not seem to be directly involved in setting up stem cell fate (Clark et al., 1996; Long et al., 1996).

The second mutant from the arrested meristem class is called *wuschel* (*wus*) (Laux et al., 1996), which is the German word for bushy. As usual, the mutant name reflects the phenotype and *wus* mutants suffer from repeated meristem termination and reinitiation, which produces plants with disorganized and bushy shoots. The *WUS* gene codes for a homeodomain transcription factor, but in contrast to *STM* it is not a member of the KNOX class, but rather the founding member of the WOX class of transcription factors (Mayer et al., 1998). *WUS* is expressed in the organizing center of the SAM, and acts in a non-cell autonomous fashion to induce stem cell

fate in the central zone. In plants lacking *WUS* activity, the SAM is depleted of stem cells, the meritematic dome collapses into a flat structure and differentiation occurs. Conversely, ectopic expression of *WUS* within the meristem causes massive overproliferation of cells (Schoof et al., 2000).

A similar overproliferation phenotype is also observed in the *clavata* (*clv*) mutants that fall into the second class of meristematic mutations (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). In plants lacking *clv* function, the meristematic dome is expanded and frequently transformed into an elongated oval shape to accommodate the proliferating cell mass, a process known as fasciation. Genetic analysis has revealed that mutations in three independent loci produce *clv* phenotypes, with *clv1* and *clv3* having the most dramatic effects. Cloning of the corresponding genes revealed that all *CLV* genes are most likely part of a single signaling pathway. *CLV1* codes for a Leucine Rich Repeat (LRR) transmembrane protein with an intracellular kinase domain (Clark et al., 1997). These proteins frequently function as plasmamembrane receptors and constitute a large family in the Arabidopsis genome. CLV2 has a similar molecular nature, but while it is also a LRR transmembrane protein, it lacks the intracellular kinase domain (Jeong et al., 1999). In contrast, the CLV3 protein is of very different structure (Fletcher et al., 1999). The active form of CLV3 is a short secreted peptide of 12 amino acids, which is processed from a longer precursor (Ito et al., 2006; Kondo et al., 2006; Rojo et al., 2002). Expression analysis of the *CLV* genes revealed that they are directly involved in plant stem cell control. *CLV3* transcripts can be found exclusively in the stem cells of the central zone, while *CLV1* RNA is restricted to a domain overlapping with the organizing center but extending partially into the stem cell domain (Clark et al., 1997; Fletcher et al., 1999). In contrast, *CLV2* is expressed more widely (Jeong et al., 1999). Double mutant analysis of *clv1* and *clv3* have indicated that both genes function in the same pathway. Further, the molecular nature of the CLV proteins indicates that CLV3 might act as a ligand for the CLV1 and CLV2 receptors. Consistent with the ligand-receptor hypothesis for the CLV pathway, CLV1 and CLV2 are known to associate in the plasma membrane (Jeong et al., 1999). So far, however, no experimental evidence for a direct interaction between CLV3 and the CLV1/2 receptors has been reported.

Taking the molecular nature and expression patterns of the *CLV* genes into account it is attractive to hypothesize that their biological function is to relay information from the stem cells to the organizing center. Based on the mutant phenotypes of all meristem regulators described above, the *CLV* pathway would relay a negative signal from the stem cells to the organizing center, while unknown molecules downstream of *WUS* would carry a positive signal in the other direction. The finding that a mutation in *WUS* is fully epistatic to all *clv* mutants has provided strong genetic support for this idea (Laux et al., 1996). Indeed, it was shown by a series of elegant experiments that *WUS* and *CLV3* are connected by a negative feedback loop (Brand et al., 2000; Schoof et al., 2000) While overexpression of *WUS* leads to ectopic formation of stem cells accompanied

by *CLV3* expression in these cells, overexpression of *CLV3* causes a strong reduction in *WUS* expression. Conversely, loss of *CLV3* function not only causes an expansion of the stem cell pool, but also the expansion of *WUS* expression in the SAM. Loss of *WUS* activity, however, leads to a loss of stem cells and, consequently, to a loss of *CLV3* expression. The model that emerged from these experiments predicted that *WUS* induces stem cell fate and *CLV3* expression, which in turn would signal back to the *WUS* expressing cells of the organizing center to limit their number (Brand et al., 2000; Schoof et al., 2000). By this simple feedback loop, the number of stem cells could be maintained by only a limited number of regulators. While conceptually very attractive, this model almost certainly an over-simplification as recent studies have shown that *WUS* and *CLV3* levels can be uncoupled without phenotypic consequences (Leibfried et al., 2005; Müller et al., 2006). Furthermore, the model is lacking a positive input and therefore the proposed system would not be able to maintain itself or to overcome perturbations.

One of the reasons why current models fail to accurately describe stem cell behavior in plants is that they are mostly based on genetic and molecular evidence obtained at the steady state level. However, cell behavior in the SAM is extremely dynamic and fluctuates over time (Reddy et al., 2004). A major step in addressing this issue was the introduction of live cell imaging for the SAM (Grandjean et al., 2004; Reddy et al., 2007; Reddy et al., 2004). Using confocal microscopy along with fluorescent reporters, it is now possible to study stem cell behavior in real time. One of the most important results of these studies so far was that stem cell identity rapidly spreads into the peripheral zone after induced knock down of *CLV3* (Reddy and Meyerowitz, 2005). Since the conversion of cell fate occurred independent of cell division, these results not only confirmed that cell fate in the SAM is based on position rather than on linage, but also that CLV3 is one of the major components of the regional signaling network. The next step to dissect the stem cell circuitry would be to visualize *CLV3* and *WUS* simultaneously to asses the role of *WUS* in the spreading of stem cell identity, which so far remains elusive.

While the genetic interaction between *WUS* and *CLV3* is well studied, the molecular underpinnings are less clear. The negative regulation of *WUS* expression by *CLV3* signaling is thought to be mediated by interaction of CLV3 with the CLV1/2 heterodimer, but so far there is no experimental evidence to support this model. However, players that act downstream of *CLV1/2* have been isolated and genetic analysis has shown that they also modulate *CLV3* signaling. Two of these downstream regulators are *POLTERGEIST (POL)* and *POLTERGEIST LIKE*, both of which code for protein phosphatase and antagonize signaling through *CLV1* and *CLV22Cs* (Song and Clark, 2005; Song et al., 2006; Yu et al., 2000, 2003). How this signal transduction cascade is linked to the regulatory elements of *WUS* (Bäurle and Laux, 2005) is so far unknown, however, as is the question of how *WUS* is able to induce stem cell fate in a subset of its neighboring cells. These questions are at the center of understanding plant meristem function.

1.1.3 Integration of Systemic and Local Signals at the SAM

After having described the local regulatory interactions governing stem cell control in the SAM, I will now discuss how systemic signals generated elsewhere in the plant can influence the apical stem cell niche. There are countless observations that highlight the importance of long distance communication for stem cell control in plants and consequently it has been thought for many years that mobile hormones must play a key role in this process. One of the simplest observations is that new meristematic centers, including their stem cells, are activated if the main shoot of a plant is removed. While it might seem trivial to any gardener that pruning stimulates growth and branching, it points to the fact that the SAM of the main shoot has the capacity to repress the development of nearby stem cell centers and that this repression is dependent on the continued presence of an active SAM. This behavior is termed apical dominance and is a major determinant of overall plant architecture (Dun et al., 2006; Ongaro and Leyser, 2007; Reinhardt and Kuhlemeier, 2002). Another basic observation is the regular arrangement, or phyllotaxis, of organs that are derived from the SAM. Be it leaves or flowers, the site of primordium initiation is carefully chosen to maximize the distance to preexisting organs (Kuhlemeier, 2007). This behavior displays the hallmarks of a reaction diffusion mechanism involving mobile signals to govern organ initiation. Genetic and molecular analyses undertaken in the last 50 years and aimed at identifying the underlying molecules and mechanisms have indeed confirmed that an array of plant hormones form the molecular basis of these processes. One of the most striking findings was that treatment of tissue explants with various plant extracts was able to stimulate dedifferentiation and proliferation of differentiated cells in vitro (Skoog and Miller, 1957). Subsequent analyses showed that a small derivative of adenine is the active agent mediating the induction of cell proliferation. It was then termed cytokinin and today is recognized as one of the most important phytohormones (Miller et al., 1955). The activity of auxin, the other hormone with a major role in stem cell control, had been reported by Charles Darwin in his paper “The Power of Movement in Plants” from 1880, in which he analyzed the light response of oat seedlings (Darwin, 1880). It turned out later that the most active form of auxin in plants is Indole Acetic Acid (IAA) and that this molecule is involved in a plethora of developmental mechanisms ranging from apical dominance to the regulation of phyllotaxis (Ongaro and Leyser, 2007; Reinhardt et al., 2003b). The importance of auxin and cytokinin for the control of stem cell fate is suggested by their activity in tissue regeneration assays *in vitro*. Pieces of fully differentiated tissue, for example from roots or leaves, will dedifferentiate and form masses of proliferating cells, or so-called callus, if treated with equal concentrations of auxin and cytokinin. That these cells indeed have stem cell characteristics is shown by the fact that they can give rise to somatic embryos or even to entire root or shoot systems if the auxin to cytokinin ratio is increased or decreased, respectively (Gamborg et al., 1968; Reinert and Bajaj, 1977). These experiments clearly demonstrate that plant cells retain their ability to be a truly totipotent stem cell even when they are fully differentiated.

This dramatic developmental plasticity contrasts sharply with the behavior of animal cells, which remain locked in their fates once differentiation has occurred.

The effects of auxin and cytokinin are not only visible during *in vitro* experiments, but are also prominent during regular plant development. In the shoot, the main function of auxin is to specify the sites of organ initiation within the SAM. In this way, auxin is the primary trigger for differentiation and consequently, mutants that lack auxin transporters, such as PINFORMED1 (PIN1) (Okada et al., 1991; Petrasek et al., 2006; Wisniewska et al., 2006), or signal transduction components, such as PINOID (PID) (Christensen et al., 2000; Friml et al., 2004), fail to initiate organs from the SAM and instead develop pin-like outgrowths. Fluorescent auxin reporter genes together with live cell imaging have shown that asymmetric localization of the auxin efflux carrier PIN1 within cells of the SAM causes transient and localized accumulation of auxin, which in turn triggers the differentiation program (Heisler et al., 2005). As mentioned above, one of the first signs of differentiation in the SAM is the repression of *STM*, and this repression is known to be mediated by auxin (Furutani et al., 2004). Interestingly, it has been shown that *STM* acts upstream of the cytokinin biosynthesis enzyme IPT (Jasinski et al., 2005). Thus, auxin might reduce cytokinin levels in organ primordia via repression of *STM*.

The antagonistic function of auxin and cytokinin in the SAM is also highlighted by phenotypes of cytokinin receptor mutants. While auxin mutants continue to grow with a severe reduction in organ initiation (Okada et al., 1991), triple mutants abolishing all three known cytokinin receptors remain very small, but are able to initiate all types of organs (Higuchi et al., 2004; Riefler et al., 2006). However, inactivation of bioactive cytokinin within the plant by overexpression of various types of cytokinin oxidases causes developmental arrest and a failure to maintain the SAM (Werner et al., 2003). A first hint how cytokinin signaling might be connected to SAM regulation on the molecular level came from the analysis of the maize mutant *Abphyll1*, which exhibits phyllotaxis defects along with an enlarged SAM (Giulini et al., 2004). The responsible gene turned out to be a member of the so-called A-type Response Regulator family, which had been described as immediate early cytokinin response genes with a role in a negative feedback of cytokinin signaling (D'Agostino et al., 2000; To et al., 2004). Thus, it seemed that increasing cytokinin signaling by removing a negative element from the signal transduction cascade caused the meristem to overgrow. This idea was confirmed when it was shown that WUS directly interacts with the regulatory sequences of the *Arabidopsis Response Regulator 7* (*ARR7*) gene to represses its expression (Leibfried et al., 2005). *ARR7* is an A-type Response Regulator and orthologue of *Abphyll1*. The same study demonstrated that interfering with cytokinin signaling by introducing a constitutively active form of ARR7 disrupts meristem function. Together with the direct regulatory interaction with WUS, this provided the first mechanistic insight into how hormonal and transcriptional signals are integrated at the SAM. In addition, local production of cytokinin also might play an important role for the activity of the SAM stem cell niche, since a mutation in *Lonely Guy* (*LOG*), a cytokinin biosynthesis gene with locally restricted expression in the meristem, causes stem cell defects in rice (Kurakawa et al., 2007).

Taken together, it seems clear from recent studies that stem cell control in the SAM is governed by a tight interplay between local transcriptional circuits and global hormonal signals. However, we are just beginning to understand how this cross talk is organized at the molecular level, and it will be exciting to learn the intricacies of this complex regulatory network in the future.

1.1.4 Differentiation – an Example from Flowers

A fascinating ability of plants is to set up stem cell populations *de novo* during regular development. The prime example for this remarkable capacity is flower formation, during which a transient pool of stem cells is induced. Upon the initiation of a floral primordium, dividing cells of the peripheral zone, which lack stem cell properties, form a bulge at the flanks of the SAM. Shortly after, when the primordium has become separated from the SAM, *WUS* expression is activated (Mayer et al., 1998), which in turn leads to the establishment of stem cells and the expression of *CLV3* in the overlaying cells in a fashion very similar to the SAM. The cells produced by the new, independent stem cell system are now used to enlarge the floral meristem and finally generate the flower. However, in order to make a flower, cells have to differentiate to take on the specialized roles required for reproduction, the function mediated by flowers. For cell differentiation to occur, stem cell fate has to be terminated and consistent with this requirement, *WUS* expression ceases about half way through the developmental program for making a flower (Mayer et al., 1998). One of the major players in mediating this termination of stem cell maintenance is the MADS box transcription factor AGAMOUS (AG) (Yanofsky et al., 1990). Flowers lacking *AG* activity not only fail to produce the proper types of floral organs, but also fail to cease production of organs after their regular number has been reached. Thus, in *ag* mutants the flower is transformed from a determinate into an indeterminate structure, which is the growth mode of the SAM. In wild type flowers *AG* is expressed in the center of young floral meristems in a domain that is largely congruent with the *WUS* domain. Interestingly, it was shown that WUS together with another unrelated transcription factor called LEAFY (LFY) (Weigel and Meyerowitz, 1993) contributes to *AG* activation in young flowers, by directly binding to *AG* regulatory sequences (Lohmann et al., 2001). In turn, one of the functions of *AG* is to repress the expression of *WUS*, since in *ag* mutants, *WUS* expression persists indefinitely (Lenhard et al., 2001; Lohmann et al., 2001). Thus, in addition to the *WUS-CLV3* feedback loop, *WUS* is engaged in a cross regulatory interaction with *AG*, which is necessary to attenuate stem cell maintenance and bring about the determinate character of flowers. Taken together, WUS plays several roles in flower development: First, it is necessary to induce a flower specific stem cell center; second, it contributes to the proper patterning of the flower via the activation of *AG;* and third, the activation of *AG* by *WUS* also causes stem cell termination. These results demonstrate a close interaction of the stem cell and patterning systems on the mechanistic level and confirm the observation made by

Johann Wolfgang von Goethe more than 200 years ago that flowers are modified shoots (Goethe, 1790).

1.2 Concluding Remarks

An active stem cell pool of defined size is maintained in the SAM, and is critical for plant development and reproduction. Under laboratory conditions, the system that controls the maintenance of the SAM is robust to a wide array of experimental conditions, and is presumably all the more so in the field. Given this enormous robustness, it is not surprising that the underlying regulatory systems are not organized as linear pathways but as a network structure. The robust nature of a functional network, however, has made dissecting the mechanistic basis of plant stem cell control challenging. In future, it will be imperative to employ multifaceted approaches and use strategies of systems biology to elucidate the regulatory logic of meristem maintenance. Although the robustness of the system makes it hard to study, the developmental plasticity of plant cells allows researchers to manipulate their fate with ease. This allows the researcher to address questions that cannot easily be tackled in animal systems.

Since stem cell niches were invented after the split of the animal and plant lineage, a comparison of the regulatory logic, as well as the molecular mechanisms involved, will give us important insights into to evolutionary constraints that led to the establishment of the stem cell systems we encounter today. Thus, the study of plant stem cell regulation can help to advance our general understanding of how stem cell systems are organized and maintained.

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Chapter 2
Stem Cell System of Sponge

Noriko Funayama

Abstract The stem cell system is one of the unique systems that have evolved in multicellular, but not unicellular, organisms. To understand the principle stem cell system in metazoans, it is important to discover the molecular and cellular mechanisms of the stem cell system in sponges (Porifera), the evolutionarily oldest living multicellular organisms. The pluripotency of sponge cells called archeocytes is suggested by basic histological studies and it is generally accepted that archeocytes are the stem cells in sponges. Germ cells are reported to originate from archeocytes or choanocytes, suggesting that choanocytes maintain pluripotency even after they have fully differentiated to have a collar, flagellum and the function of nutrient entrapment. Recently, increasing molecular biological studies of sponges have been reported, including the identification of lineage-specific molecular markers. The stem cell system of sponge is discussed here based on both histological and molecular biological studies.

Keywords Sponges, Porifera, stem cell, archeocyte, choanocyte

2.1 Stem Cell System in Sponge, The Evolutionarily Oldest Metazoan

If we think about the evolution from unicellular to multicellular organisms at the cell level, we realize that a key point is that a unicellular organism must do everything by itself, including proliferation, nutrient intake, defense and so on, whereas in the multicellular organism, cells can share these tasks and simultaneously develop systems to coordinate and interact with each other. The stem cell system is one of

Laboratory of Molecular Developmental Biology, Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan
Tel.: + 81-75-753-3649, Fax: + 81-75-753-4200
E-mail: funayama@mdb.biophys.kyoto-u.ac.jp

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the unique systems that multicellular organisms have evolved. Sponges (phylum Porifera) are the most basic animals among multicellular organisms. Although we don't know the actual origin of metazoans, sponges should provide clues for understanding the most basic stem cell system in metazoans. In addition, comparing the stem cell system of sponge to those of animals in different phyla and those of plants would enable us to think about how the stem cell system evolved, which parts of this system have been conserved throughout evolution and which parts are specialized in certain phyla.

2.2 Cell Types Constructing the Sponge Body

Porifera is a large phylum that includes many species with diverse morphologies and includes the classes Demospongiae, Calcarea, and Hexactinellida. In this chapter, I focus on studies using cellular sponges, Demosponges and Calcareas, as Hexactinellids consist largely of a single syncytial tissue (for review of Hexactinellida see Leys et al., 2007). The body of a cellular sponge can be roughly divided into three parts (Fig. 2.1): outer epithelial cells (including basal epithelial cells) that cover the sponge body, inner epithelial cells constructing canal systems, and mesohyl, the space between the outer and inner epithelial cells. The mesohyl includes many types of cells, collagen fibrils, and spicules.

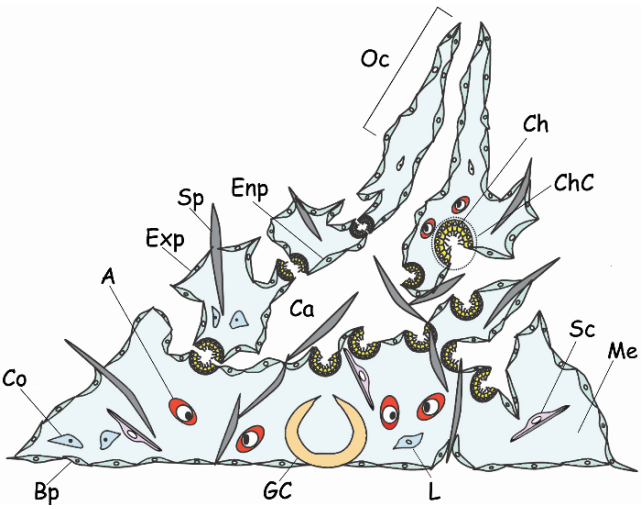

Fig. 2.1 Vertical sectioned view of gemmule-hatched juvenile of *Ephydatia fluviatilis*. A; archeocyte, Bp; basopinacocyte, Ca; water canal, Ch; choanocyte, ChC; choanocyte chamber, Co; collagenocyte, Enp; endopinacocyte, Exp; exopinacocyte, GC; gemmule coat, L; lophocyte, Me; mesohyl, Oc; oscule, Sc; sclerocyte, Sp; spicule. (Modified from Funayama et al. (2005a) Develop. Growth Differ. 47, 243–253, Blackwell)

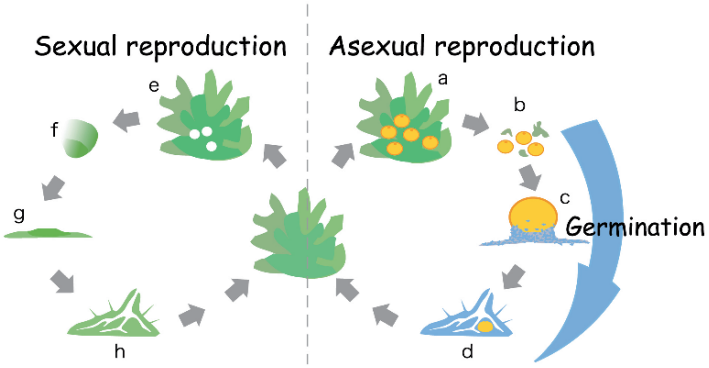

Fig. 2.2 Life cycle of *Ephydatia fluviatilis*. In asexual reproduction, (a) Gemmules are formed within the parent sponge tissue. Thousands of resting stem cells are contained within the gemmule coat. (b) When the parent sponge is destroyed by high temperature or dry conditions, parent sponge tissue around the gemmule is eliminated, and then hatching occurs. (c) Cells migrate out from the gemmule and, (d) form a fully functional small sponge within 1 week. The stages shown in (b) through (d) are collectively called "germination". In sexual reproduction, sperm and eggs are formed within the male or female sponge, respectively. (e) Eggs are fertilized within the female sponge tissue and begin cleavage. (f) Flagellated swimming larvae leave the parent sponge and then (g) settle and start to metamorphose. (h) A new generation of sponge is formed where the sponge has settled. (Funayama et al. (2005a) Develop. Growth Differ. 47, 243–253, Blackwell)

More than 10 types of cells have been reported to make up the sponge body (Fig. 2.1). Most of these cells have been defined only by their morphological features. Stem cells are known as archeocytes. Thesocytes are archeocytes in the resting state in the gemmule of sponges undergoing asexual reproduction (Fig. 2.2). Choanocytes are food-eating cells. Each choanocyte has a collar and a single flagellum. Choanocytes form a single-layered epithelial sphere, called a choanocyte chamber. By moving their flagella, they create water currents that help them to take up nutrients from the outside. Sclerocytes are the spicule-making cells. The outer epithelial cells are known as exopinacocytes, and the inner epithelial cells as endopinacocytes. Basal epithelial cells are known as basopinacocytes. Sclerocytes are the spicule forming cells. Additionally, spongocytes, collagenocytes and lophocytes are active in collagen biosynthesis. There are more types of cells that have been reported in many but not all sponge species (e.g., myocytes as contractile cells, glycocytes as cells involved in the storage of glycogen, and spherulous cells with many inclusions) (Simpson, 1984; Boury-Esnault, 2006).

2.3 Archeocytes as Pluripotent Stem Cells

In this chapter, the terms 'totipotent' and 'pluripotent' are used according to the review of Seydoux and Braun. Totipotency is the ability of a single cell to divide and produce all the differentiated cells in an organism. This potential is maintained

in the female germline throughout development. Pluripotency is the ability of a single cell to produce differentiated cell types (Seydoux and Braun, 2006).

Harrison described the archeocytes as morphologically similar to the unspecialized stem cells in other animals (i.e., they have single nucleolated nuclei) (Harrison, 1974). Archeocytes are defined as large amoeboid cells that have nuclei with a large single nucleolus. Archeocytes actively migrate within the mesohyl and are highly proliferative and phagocytic cells.

Based mostly on histological studies, it is generally agreed that the archeocytes are likely to be the pluripotent stem cells in sponge, and other types of cells originate from archeocytes (Borojevic, 1966, 1970).

On the other hand, there are some reports that choanocytes can transdifferentiate into archeocytes, germ cells or even epithelial cells on specific occasions (Connes et al., 1974; Diaz, 1977; Buscema and Van de Vyver, 1979; Simpson, 1984), suggesting that choanocytes maintain pluripotency in spite of having specialized functions and specialized cell morphology. The pluripotency of choanocytes will be discussed in Section 2.10.

2.4 Archeocytes in Growth Area

Brien reported that archeocytes migrate into the new growth area in gemmule-hatched juveniles of the freshwater sponge *Ephydatia fluviatili*s or in the tips of the branches of the adult freshwater sponge *Spongilla lacustris*, and archeocytes may proliferate and differentiate into specialized types of cells, including choanocytes and sclerocytes, two cell types that can be easily distinguished even under the light microscope (Brien, 1976). Archeocytes differentiate into most of the fundamental types of cells, pinacocytes, spongocytes, lophocytes, choanocytes and sclerocytes, present in the growth area (Simpson, 1984).

2.5 Archeocytes in Remodeling and Reconstitution

Sponges undergo remodeling routinely a) to refine their canal systems, b) to reconstruct tissues, including canal systems, after degeneration of tissue during sexual reproduction, and c) to functionally connect newly grown areas with the original canal systems. In all cases, archeocytes phagocytose other cells and develop numerous phagosomes that are strikingly similar to those in gemmular thesocytes, and then differentiate into other types of cells, including choanocytes and endopinacocytes, that develop to form canals (Diaz, 1979; Simpson, 1984).

It is well known that sponges have remarkable regenerative and reconstitutive abilities. Sponges were the first metazoans in which the reconstitution of dissociated cells was demonstrated (Wilson, 1907). In the usual regeneration and reconstitution

processes, dissociated cells settle onto the substrate, and cells and small cell aggregates actively migrate and coalesce to form larger aggregates. When the aggregate becomes a certain size, it attaches to the substrate and elongates. The elongated aggregate then develops into a functional sponge.

In the initial aggregates, cells are mixed randomly but at least choanocytes have been reported to become sorted out from other cells (Van de Vyver, 1975). In *E. fluviatilis*, cell division of archeocytes in aggregates is substantially increased compared to that in the intact sponge, but cell division of anucleolate mesohyl cells is not detected (Efremova, 1970). Buscema et al. clearly showed that archeocytes differentiate into choanocytes and pinacocytes in aggregates, as judged by their distinct ultrastructural differences (Buscema et al., 1980). The pluripotency of the 'adult' archeocytes was demonstrated by their work. Archeocytes were reported to play a significant role in the reconstitution of the dissociated sponge, as the dissociated cells without the archeocyte-rich fraction could not reconstitute the sponge body (De Sutter and Van de Vyver, 1977, 1979). On the other hand, aggregates of cells that are isolated as archeocyte-rich fractions are able to develop into functional sponges (De Sutter and Van de Vyver, 1977; De Sutter and Tulip, 1981, Funayama et al. unpublished data). Although the above findings strongly suggest the pluripotency of archeocytes, experiments using molecular markers of membrane-spanning proteins of archeocytes to isolate a 100% pure archeocyte fraction or a single cell culture technique for archeocytes will be required to obtain conclusive results.

There have been numerous studies in which the histology of aggregates was examined in order to determine the origin of some types of newly formed cells. There are some discrepancies among these studies, probably due to differences of the species, the physiological condition of the sponges and/or experimental conditions (Simpson, 1984). These studies suggest that reconstitution of the sponge body from dissociated cells is not simply like gathering the dissociated colorful blocks to reconstruct a Lego house; rather, it is a remodeling process in which some types of cells retain their features and are reused in reconstitution, whereas simultaneously some types of cells degenerate or are phagocytosed, and then new cells of that type are produced by differentiation from archeocytes.

2.6 Origin of Germ Cells

In sexual reproduction (Fig. 2.2), germ cells are formed within the mesohyl and eggs are either fertilized *in situ* in the mesohyl and then undergo development or are released and undergo development in the water (Simpson, 1984, for embryogenesis see Leys and Ereskovsky, 2006). Germ cells are known to differentiate from archeocytes or choanocytes. Oocytes have been suggested to differentiate directly from archeocytes in many species (Simpson, 1984), and from choanocytes in the demosponge *Suberites massa* (Diaz et al., 1973, 1975; Diaz, 1979), in Calcarea sponges (Sara, 1974; Gallissian, 1981; Franzen, 1988), and freshwater sponges of Lake Baikal (Ereskovsky, 1999) among others.

In *S. lacustris,* archeocytes enlarge by two steps to become oogonia. In the first phase of growth, soluble materials are taken up, and the second phase involves the phagocytosis of trophocytes or other cells (Leveaux, 1941). Phagocytosis of other cells by oocytes has been reported both in species that have oocytes that originate from archeocytes, and in species that have oocytes of choanocyte origin.

Among the species in which oocytes have been suggested to be derived from choanocytes, Calcarea, *Scypha ciliata* (Franzen, 1988) and freshwater sponges of Lake Baikal, family Lubormirskiidae (Ereskovsky, 1999) have been studied in the greatest detail regarding oogenesis using light and electron microscopy. In *S. ciliata*, the youngest oocytes which are just beginning to develop are always found in the mesohyl below the choanocytes. It was reported that these oocytes are derived from choanocytes, as inferred from their close contact with the overlying choanocytes, and the similarity of the cytoplasmic structure of oocytes and choanocytes (Franzen, 1988).

Spermatocytes have been suggested to be derived from choanocytes in several species, for example, in *Aplysilla rosea* (Tuzet et al., 1970a,b), and in *E. fluviatilis* (Paulus and Weissenfels, 1986), *S. lacustris* (Paulus, 1989). Detailed electron microscopic studies of Tuzet et al. strongly suggested that spermatocytes originate from choanocytes, as the flagella of choanocytes are maintained during spermatogenesis. Choanocytes forming a single chamber simultaneously transform into spermatogonia, losing their collar and phagosome-containing posterior cell region but retaining their flagellum. Spermatogonia are surrounded by newly formed epithelia and spermatogenesis proceeds within this follicle. Primary spermatocytes develop directly from spermatogonia, and then secondary spermatocytes develop by meiosis. Finally, a follicle including mature sperm is formed.

As all the observations described above were made on fixed material, experiments using the cell lineage tracing technique will be needed to prove the origin of germ cells. It is worth noting that due to the ability of choanocytes to produce germ cells, choanocytes should maintain their pluripotency even after they become fully differentiated from archeocytes.

2.7 Archeocytes in Asexual Reproduction

2.7.1 Bud Formation

Some species of sponge develop and release buds as a form of asexual reproduction. In the pioneering work of Connes, the process of budding in *Tethya lyncurium* was described in detail (Connes, 1967, 1968). Typically, the distal tips of the filaments of the sponge body swell and round up to form buds. Eventually, buds are pinched off and become free floating, and then they eventually attach to a new substrate. The principal cell types present in the mature bud are archeocytes. It is thought that after attachment, archeocytes differentiate to form a new functional sponge (Connes, 1967; Boury-Esnault 1970; Simpson, 1984).

2.7.2 Gemmule Formation

Some species of sponge, including the freshwater sponge *E. fluviatilis*, used in our studies, have both sexual and asexual reproduction systems (Fig. 2.2). Asexual reproduction in the sponge begins with the formation of small particles, known as gemmules, within the parent sponge tissue. The cells that ultimately constitute fully formed mature gemmules are referred to as thesocytes and are, in fact, resting archeocytes that contain numerous complex vitelline platelets (in freshwater sponges) or similarly complex storage granules (in marine sponges). The thesocytes of *E. fluviatilis* are binucleate and have cytoplasm packed with vitelline platelets (Simpson, 1984).

Gemmule formation has been studied in a number of species, especially in *E. fluviatilis* (Rasmont, 1956; Simpson, 1984; De Vos, 1971, 1977; Langenbruch, 1981; Mohri and Funayama unpublished data), as gemmule formation can be induced *in vitro* (Rasmont, 1974). Specific stages in the process of gemmule formation have been proposed based on the time-lapse microscopic study of Rasmont and De Vos (1974). In the initiation of gemmule formation, archeocytes, trophocytes and spongocytes are reported to accumulate locally (De Vos, 1971, 1977; Langenbruch, 1981). All three types of cells share common features: a large nucleolate nucleus, numerous Golgi bodies, abundant ribosomes and mitochondria. Trophocytes contain numerous membrane-bound lipid inclusions and numerous free ribosomes. Spongocytes are distinguished by exceptionally well-developed rough endoplasmic reticulum and are thought to secrete collagen to form the shell of the gemmule, as the so-called “gemmule coat”, in later stages of gemmule formation. It is still not clear whether these three types of cells are already differentiated when they join the aggregate, differentiate during the process of joining the aggregate, or differentiate after joining the aggregate.

The aggregate grows by adding more of these mesohyl cells. In an electron microscopic study, phagocytosis by archeocytes of the cytoplasmic portion, including lipid inclusions, of trophocytes was detected. In the aggregate, archeocytes mature to form platelets using these nutrients from trophocytes. Eventually, aggregates of archeocytes are surrounded by two layers consisting of flattened epithelial cells and spongocytes (Langenbruch, 1981). The gemmule coat is formed by the collagen secreted by spongocytes around the aggregates and embedding of the gemmule spicules produced by gemmulosclerocytes. Around the end of the stage of formation of the gemmule coat, platelet-laden internal archeocytes become binucleate (thesocytes). How this occurs has not yet been described.

2.7.3 Hatching from Gemmules

The germination (hatching from gemmules) process, starting with hatching from the gemmule, is thought to be a system to ensure survival during dry or high-temperature

conditions that can severely damage the parent sponge. Usually, germination is inhibited by an inhibitory factor from the parent sponge tissue, but when the parent sponge is destroyed and inhibitor(s) are subsequently eliminated from around the gemmule, germination occurs (Simpson, 1984).

In *E. fluviatilis*, when germination begins, thesocytes undergo mitosis within the gemmule coat and become either archeocytes (stem cells, mononucleate) or histoblasts (with nuclei lacking a nucleolus and few, if any, platelets) (Simpson, 1984). Histoblasts and archeocytes then migrate out from the gemmule coat. Archeocytes proliferate and differentiate into all types of cells to form a fully functional miniature sponge (Höhr, 1977). The histoblasts are thought to become the basopinacocytes (basal epithelial cells) in the early stages of germination. The histoblasts might be archeocytes/thesocytes committed to the basopinacocyte-lineage. It is not clear whether the histoblast directly differentiates from the thesocyte or from the archeocyte derived from the thesocyte.

As it is thought that all types of sponge cells are differentiated from the archeocyte during germination, germination can be viewed as a good system to investigate the regulation of stem cell differentiation in the evolutionarily oldest metazoan. However, until recently most of the work on sponge archeocytes has been limited to observational studies. To understand archeocyte differentiation more deeply, we need to understand it in molecular terms.

2.8 A Major Technical Problem in Sponge Cell Biology

One of the major problems in sponge cell biology studies is that the cell types are classified mostly by their cell morphology and most of cells in the mesohyl are morphologically similar. In spite of the substantial number of intense studies describing cell types in certain tissues and studies attempting to shed light on the origin of particular types of cells, it is difficult to draw definite conclusions from them.

There are two major problems in the identification or classification of the types of cells in sponges. One is the presence of cells that are morphologically intermediate between archeocytes and other differentiated cells, presumably archeocyte-derived cells committed to a particular cell lineage. The other is that, as archeocytes have both pluripotency and high phagocytic activity, some of the previously described classes of cells (such as Gray cells or spherulous cells) are probably archeocytes actively engaged in the phagocytosis of degraded cells as a source of nutrients. In addition, as Porifera is a big phylum, there must be some diversity in morphology among cells of the same type.

For the next step in sponge cell biology, we need to perform molecular biology studies, such as the identification of cell types using cell type-specific molecular markers, and the development of a gene transfer technique to resolve the functions of genes and perform cell-lineage tracing.

2.9 Molecular Studies on Stem Cell System of Sponge

2.9.1 Cell Lineage-Specific Molecular Markers

To clarify the mechanisms involved in regulation of archeocyte differentiation, we have focused recently on the asexual reproduction process of sponges, known as germination. As all types of cells originate from archeocytes in germination, this can be a very unique and useful system to investigate the processes and regulation of stem cell differentiation in the evolutionarily oldest metazoan. Among sponge species that have an asexual reproduction system involving gemmules, we selected the freshwater sponge *E. fluviatilis* for our studies as gemmules of this species are exceptionally able to hatch in the laboratory throughout the year. Hatching from the gemmule in most other species is limited to a particular season of the year.

As written in Section 2.2, because most of the cell types of sponge are defined by their cell morphology, it has been difficult to distinguish archeocytes, differentiating archeocytes and fully differentiated cells with archeocyte-like features, such as a nucleus with a single nucleolus. In order to overcome this problem and to understand the processes of stem cell differentiation in sponges at the molecular level, we first attempted to isolate cell lineage-specific molecular markers of *E. fluviatilis*.

2.9.2 Sclerocyte-Lineage-Specific Marker

As possible sclerocyte-lineage markers, we focused on silicateins, which are sponge enzymes that function to deposit silica in the core filament of siliceous spicules (Shimizu et al., 1998) The Ef silicatein gene was isolated from our EST library of juvenile sponges that had hatched from gemmules of *E. fluviatilis* by BLAST search (Funayama et al., 2005b). Ef silicatein shares 45% to 60% homology with silicateins of other sponges (Shimizu et al., 1998; Zhou et al., 1999; Cha et al., 1999; Krasko et al., 2000; Schröder et al., 2003; Uriz, 2006; Mohri et al., 2008). The pattern of Ef silicatein expression reveals the developmental stages of spicule formation: Ef silicatein was found to be expressed in round cells without spicules that were presumably archeocytes committed to the sclerocyte-lineage or else sclerocytes before spicule formation, sclerocytes containing immature spicules, and sclerocytes containing spicules of almost the same size as mature spicules (Fig. 2.3A–C). At the final stage of development, the sclerocyte punctures and rounds itself off from the mature spicule.

In the marine sponge *Suberites domuncula*, the expression of genes that have homology to the mesenchymal stem cell-like protein (MSCP-1) and noggin family genes is increased in primmorphs, proliferating and differentiating cells in spheroids when inorganic morphogenetic factors silicon and ferric ion are added to the culture medium (Custodio et al., 1998; Krasko et al., 2002; Müller et al., 2004; Müller, 2006). Noggin was originally identified by its dorsalizing activity in

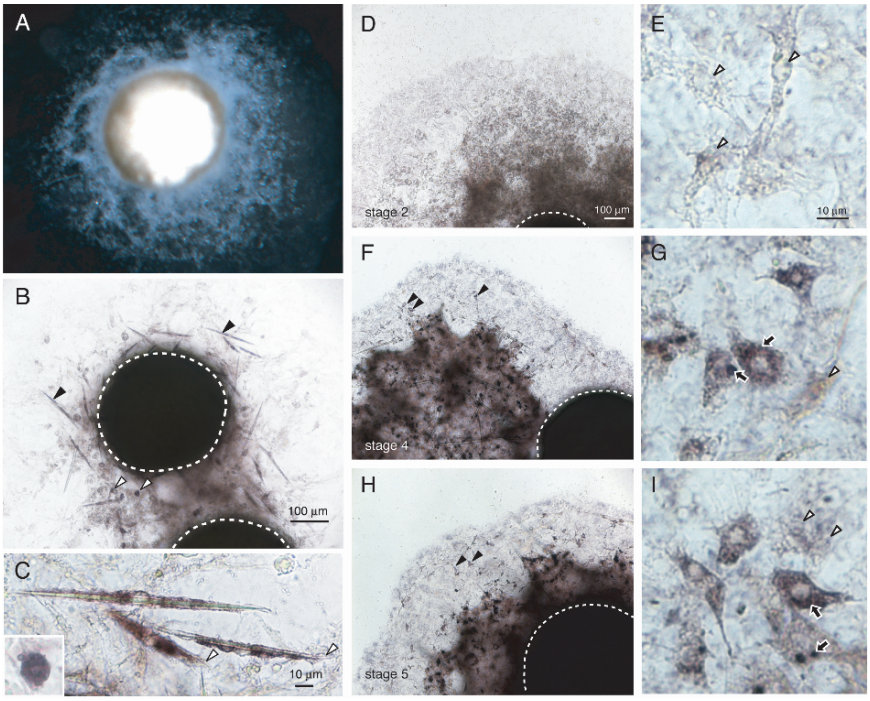

Fig. 2.3 *Ef silicatein* expression in the sclerocyte lineage (A–C) and *Ef lectin* expression during hatching from the gemmule (D–I). Whole mount *in situ* hybridization of *Ef silicatein* in stage 2. (A) A bright-field image of a stage 2 sponge. (B) *Ef silicatein* expression is detected in sclerocytes forming spicules (black arrowheads), and in round cells (white arrowheads). (C) At higher magnification, developing spicules can be detected inside each elongating sclerocyte. Sclerocytes in the early stage of spicule formation have short, thin, immature spicules. Note that the spicules are slightly bent, indicating that they are still immature, with little silica deposited (C, white arrowheads). A round cell that is expressing *Ef silicatein* and that contains a relatively large amount of yolk granules, presumably an archeocyte committed to become a sclerocyte (C, insert).
Whole mount *in situ* hybridization of *Ef lectin* at low magnification (D, F, H) and high magnification (E, G, I). *Ef lectin* is not expressed at early developmental stages, including stage 2 (D, white arrowheads in E). In fully developed sponges (F) that are maintaining and gradually expanding their body (H), cells expressing *Ef lectin* are detected (black arrowheads). *Ef lectin*-positive cells are distributed throughout the sponge body (F, H), are relatively large in size, and have a flattened shape with extensions (G, I). *Ef lectin*-negative cells in late developmental stages are indicated by white arrowheads (G, I). Examples of possible mRNA localization suggested by dot-like signals (black arrows). (Funayama et al. (2005b) Zool. Sci. 22:1113–1122, J-STAGE)

Xenopus embryos. Noggin binds to BMPs to antagonize their activities. A noggin-like protein is a candidate for a signaling molecule that regulates the commitment of archeocytes to the sclerocyte lineage, or sclerocyte/spicule maturation. Further investigations will be needed to reveal which type of cells express noggin-like proteins and the function of these noggin-like proteins.

2.9.3 Molecular Marker for Cells Presumably Involved in Innate Immunity

The Ef lectin gene was isolated by a BLAST search of our EST library (Funayama et al., 2005b). Ef lectin is highly homologous to a special type of lectins of marine sponges and the horseshoe crab. Both proteins are reported to bind to the surface of bacteria and inhibit bacterial proliferation. We speculated that Ef lectin is expressed in cells that act as a defense against invading bacteria. Ef lectin is not expressed in the early developmental stages of juveniles hatched from gemmules, but is expressed in the later developmental stages. This expression pattern suggests that Ef lectin is expressed in differentiated cells. Ef lectin-expressing cells were observed to be relatively large and distributed throughout the sponge body, and tended to be localized beneath the epithelial cells (Fig. 2.3D–I).

2.9.4 Choanocyte-Lineage-Specific Marker

We isolated Ef annexin as a cell-lineage-specific marker for choanocytes (Funayama et al., 2005a), using specific labeling of choanocytes with fluorescent beads followed by FACS sorting and a proteomic approach. We developed a high resolution *in situ* hybridization technique that can detect gene expression at the cellular level in juveniles of freshwater sponges (Fig. 2.4). Using this technique, *Ef annexin* was shown to be expressed from an early stage of choanocyte differentiation until the stage when choanocytes form mature choanocyte chambers, including cells presumed to be archeocytes committed to the choanocyte lineage, choanocytes/choanoblasts forming 2-, 4-, and multiple-cell clusters, and choanocytes forming choanocyte chambers of mature size. This expression pattern of *Ef annexin* suggests that archeocytes committed to choanocytes undergo several cell divisions to produce choanocytes that form a single choanocyte chamber (Funayama et al., 2005a).

2.9.5 Likely Candidates for Molecular Markers for Particular Types of Cells

In *S. domuncula,* several genes are reported to be expressed in particular types of cells. In primmorphs, spherical cell aggregates produced in vitro, one homeobox gene, Iroquois, is expressed in cells adjacent to the canal system (presumably endopinacocytes) and in very scattered cells in the cortex region (Perovic et al., 2003). In *S. domuncula,* during the sexual reproduction season, expression of one of the receptor-type tyrosine kinases (RTKvs_SUBDO) in oocytes and in larvae of morula and mature stages was detected using polyclonal antibody raised against the extracellular domain of RTKvs_SUBDO protein (Perovic-Ottstadt et al., 2004).

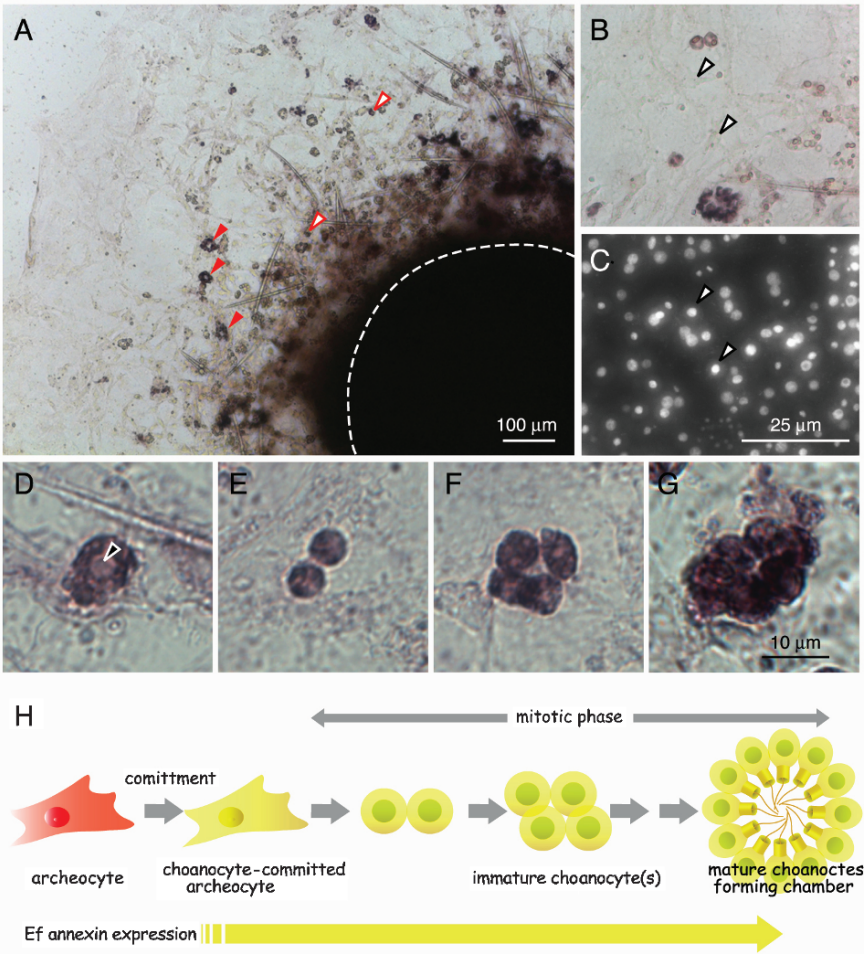

Fig. 2.4 Development of choanocyte chambers can be visualized by *Ef annexin* expression. Whole mount *in situ* hybridization of stage 2 (4 day old) sponge. (A) *Ef annexin*-expressing single cells are indicated by red-bordered white arrowheads. Solid red arrowheads indicate multiple-cell clusters. The gemmule coat is indicated by a white dashed line. (B,C) Many Ef annexin-negative cells are shown. (B) *In situ* hybridization for Ef annexin mRNA, and (C) Corresponding Hoechst staining. Black-bordered white arrowheads show typical examples of Ef annexin-negative cells. (D-G) Higher magnification of developing choanocyte chambers. *Ef annexin* is expressed in: (D) Rather large single cells with a nucleolated nucleus (white-bordered black arrowhead) containing many yolk platelets, presumably choanocyte-committed archeocytes; E) 2-cell clusters; (F) 4-cell clusters; (G) spherical clusters. (H) Model of archeocyte to choanocyte differentiation. Archeocyte commits to the choanocyte lineage and starts to express *Ef annexin*. The archeocyte committed to the choanocyte lineage undergoes mitosis to produce a 2-cell pair of choanocytes. A 4-cell cluster of choanocytes is formed by another round of mitosis. After several cell division cycles, choanocytes form a mature chamber. (Funayama et al. Develop. (2005a) Growth Diff. 47, 243–253, Blackwell)

2.9.6 Genes Expressed in Archeocytes

We have recently isolated genes expressed in archeocytes (Funayama unpublished data). One of these genes has been designated Ef piwi because it contains domains that show homology with the PIWI and PAZ domains of piwi genes. Piwi genes are known to be expressed in germline stem cells or stem cells in other animals and plants and to function in the maintenance of pluri/toti/multi-potency by RNA-dependent mechanisms. Recently, our knowledge about piwi genes is increasing; in mammals and flies, piwi proteins bind to small RNAs (24–27 nucleotides, designated as piRNAs) but the pool of piRNAs is very complex. Several functions of piwi have been suggested, including the degradation of sense transposon transcripts, the production of antisense repeat-associated siRNAs, and taking part in transcriptional gene silencing processes involving polycomb proteins in flies (for reviews see Lin, 2007; Seto et al., 2007; O'Donnell and Boeke, 2007). It is very intriguing that archeocytes express Ef piwi, suggesting that the mechanism that maintains pluri/toti/multi-potency in a microRNA-PIWI-dependent manner might have already been developed at a very early stage of evolution of Metazoans.

2.10 The Process of Archeocyte Differentiation and the Stem Cell System of *E. Fluviatilis* Revealed Using Molecular Markers

By analyzing the expression of Ef piwi and specialized cell-lineage marker genes using dual color *in situ* hybridization, it was clearly demonstrated that choanocytes, cells that are expected to have roles in innate immunity, and sclerocytes are all differentiated from archeocytes (Funayama, unpublished data). Furthermore, it was revealed that there are two different modes of differentiation of archeocytes. During the process of archeocyte differentiation, Ef piwi expression ceases, while the differentiation marker genes start to be expressed. We think this type of gene switching generally occurs during the process of archeocyte differentiation. However, unexpectedly we found that Ef piwi expression is maintained exceptionally in the case of differentiation into choanocytes. That is, Ef piwi expression is maintained even in the mature choanocytes. As described in Section 2.6, it has been suggested that spermatocytes of *E. fluviatilis* originate from choanocytes (Paulus and Weissenfels, 1986). Considering the Ef piwi expression pattern and known functions of piwi proteins together with this work of Paulus and Weissenfels strongly suggests that at least choanocytes of *E. fluviatilis* maintain pluripotency. In addition, it was suggested that choanocytes are transformed into archeocytes during remodeling/regeneration in *Suberites massa* (Diaz, 1977, 1979) and during gemmule formation in *S. domuncula* (Herlant-Meewis, 1948; Connes, 1977). Transformation of *E. fluviatilis* choanocytes into pinacocytes via the retraction of their collars and flagella in aggregates of dissociated cells has been reported (Buscema and Van de Vyver, 1979). The ability

of choanocytes to exercise the potential of pluripotency is assumed to be regulated and to require some signals. Aggregates in a mixture of choanocyte and pinacocyte fractions show no attachment to the substrate or further development (De Sutter and Van de Vyver, 1977; Simpson, 1984).

The potential pluripotency of choanocytes seems reasonable for the following reasons. First, for differentiation into specialized cells, stem cells require a sufficient amount of energy. This may be one of the reasons for the high phagocytic activity of archeocytes. As the main function of choanocytes is nutrient intake, choanocytes might also be able to play a role as a second class of stem cells in sponge. Second, choanocytes are morphologically similar to the Choanoflagellata, which have been suggested to be one of the closest unicellular organisms to multicellular organisms based on studies of gene variation and phylogenetic studies. The fact that some species of choanoflagellates aggregate suggests that the prototype of the multicellular organism might have developed from an aggregate of choanoflagellata/choanocyte-like cells. The pluripotency of choanocytes is in accord with this possibility.

In summary, it has been demonstrated that the stem cell system in sponge includes two types of pluripotent stem cells, archeocytes and choanocytes (Fig. 2.5). Both types of cells have the ability to differentiate into somatic cells and germ cells. For generating somatic cells, archeocytes play a major role by differentiating into all types of somatic cells, including choanocytes. The second type of pluripotent stem cells, choanocytes, appear to function as a storage system for stem cells that can transform mainly into archeocytes, which then produce other types of cells. It should be noted, though, that there are several reports that choanocytes differentiate directly into somatic cells under special conditions.

Both archeocytes and choanocytes have the ability to differentiate into germ cells. However, which type of cells transform into oogonia or spermatogonia is different depending on the species.

2.11 Interesting Issues Regarding the Stem Cell System of Sponge To Be Addressed in Future Investigations

(a) The mechanisms of maintaining and regulating the pluripotency of archeocytes and choanocytes. The extrinsic or intrinsic signals that control stem cell maintenance, the retention of pluripotency and the regulation of the proliferation of stem cells, have been investigated in various species. For example, BMP signaling and Piwi are suggested to have important roles in Drosophila germline stem cells (for review see Megosh et al., 2006). The Ef piwi expression in archeocytes and choanocytes is a good first clue for elucidating the molecular mechanisms of the maintenance of pluripotency in sponge.

It will be of great interest to determine whether choanocytes maintain their pluripotency using the same molecular mechanism as archeocytes, to understand how the pluripotency of choanocytes is suppressed in the normal situation, and how the pluripotency of choanocytes is exercised in cellular and molecular terms.

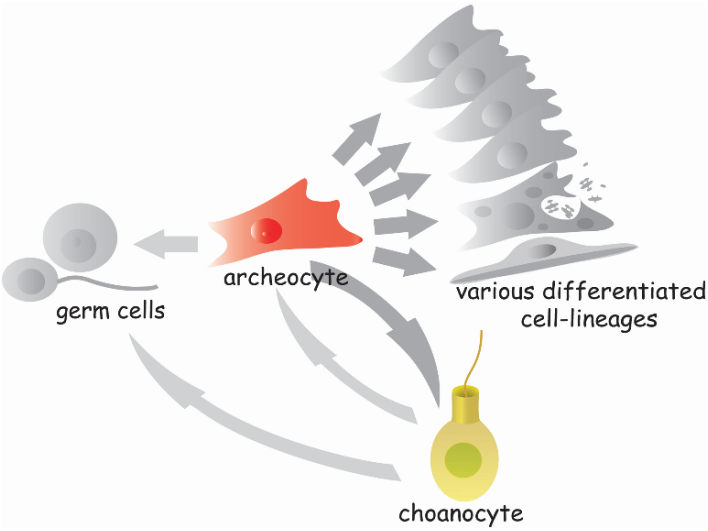

Fig. 2.5 Simplified model of the stem cell system of sponge. Archeocytes, the pluripotent stem cell, differentiate into all types of cells including choanocytes, sclerocytes, cells that act in innate immunity, and germ cells. The special features of the stem cell system of sponge are that: (1) archeocytes also act as active phagocytes, probably to gain energy for differentiation. (2) The presence of a second type of cell, choanocytes, that maintain pluripotency although they have specialized functions of entrapping nutrients and specialized morphology with a single flagellum and collar. Choanocytes are suggested to maintain pluripotency, as indicated by their ability to transform into germ cells or archeocytes under specific conditions. There are some reports suggesting that choanocytes can be transformed directly into pinacocytes or other types of cells. Choanocytes might function as a store of stem cells. Although the available data strongly suggest that choanocytes can be transformed into archeocytes, germ cells or other types of cells, this should be confirmed using cell lineage tracing. The major challenge in understanding the stem cell system of sponges is elucidating the cellular and molecular mechanisms regulating the differentiation/transformation of the two types of pluripotent cells

(b) Cell division, including the process of archeocyte differentiation. One very important issue is whether archeocyte differentiation is accompanied by cell division. There are three possibilities if cell division is involved in this process: archeocytes differentiate into certain types of cells without cell division, with asymmetric cell division to produce archeocytes and certain types of cells, and with symmetric division to produce two differentiated cells. The type of process might be different depending on the cell lineage.

(c) The molecular mechanisms of the induction of differentiation of archeocytes into certain types of cells. Using lineage-specific molecular markers, it has become possible to detect the processes of archeocyte differentiation into the several cell lineages for which such markers are available. Studies using these markers will make it possible to answer questions such as: Is adhesion to or contact with particular types of cells involved? Are there secreted signaling molecules that induce differentiation of archeocytes into specific types of cells? Wnts and small

peptides, including amidated peptides, are reported to have roles in epithelial stem cell differentiation in Hydra (for review see Bosch, 2007). These are some of the candidates for secreted molecules that regulate archeocyte differentiation.

(d) The resting stem cells. Adult/tissue stem cells animals in various phyla are usually in a resting state. Recent studies of pluripotent stem cells in planarian also suggest that there is a subpopulation of stem cells in a very slow cell cycle or resting state (Shibata, unpublished data). The resting state of stem cells is considered to have developed to save stem cells from depletion, and simultaneously to protect the host from over proliferation of stem cells (for review of the stem cell niche see Xie and Li, 2007; Scadden, 2006). Although sponges always tend to increase their body size if they are under good conditions, and thus their archeocytes are always active, there should be some subpopulation of archeocytes in a resting state. It will be very interesting to investigate the resting or very slow cell cycle archeocytes in adult sponges, and the microenvironment, the niche, that regulates the resting state of archeocytes. It is possible that choanocytes function as resting stem cells in adult sponges.

Thesocytes in the gemmule are dormant in most species, or in diapause in several species, including *E. fluviatilis*. In the sense that resting stem cells in other organisms are ready to activate, thesocytes in diapause gemmules would be in similar state with resting stem cells in other organisms. It is important to clarify and compare both mechanisms that regulate thesocytes in dormant and diapause gemmules. It will give us clues to understand mechanisms that regulate thesocytes into a very tight resting state (in dormant gemmules) or into a ready to activate resting state (in diapause gemmule). Furthermore, whether there are some similarities between the mechanisms that regulate thesocytes in diapause gemmules and resting stem cells in other organisms is a very absorbing issue. We have started studies on gemmule formation in *E. fluviatilis* as a unique cutting edge system to investigate the cellular and molecular regulatory system of transformation from active archeocytes into resting archeocytes, thesocytes. Novel insights into both the intrinsic and extrinsic regulatory systems of resting stem cells are expected.

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Chapter 3
Stem Cells in Immortal *Hydra*

Thomas C. G. Bosch

Abstract *Hydra*'s potential immortality and extensive capacity to regenerate and self-renew is due to the presence of three distinct stem cell lineages: ectodermal and endodermal epithelial stem cells, and interstitial stem cells. Over the last few years, stem cells in *Hydra* became well-defined in cellular terms of their biology. More recently, efforts using the nearly unlimited potential for tissue manipulation combined with functional transgenesis have shed light on the molecular control mechanisms involved. Here I review those efforts in an attempt to give both a historical perspective and an update on the recent experimental highlights. In particular, I will focus on six aspects of stem cells in Hydra: (i) their continuous transition through the proliferation/differentiation switch; (ii) their rapid responses to signals from the cellular environment; (iii) the emerging importance of Wnt and Notch signaling in controlling stem cell behavior; (iv) the role of chromatin modification in terminal differentiation; (v) the observation of transdifferentiation in some of the stem cell progeny; and (vi) the implications for the evolution of germ cells, ageing and cancer. Together, these findings seem to indicate that *Hydra* not only provides insights into signalling pathways involved in stem cell differentiation in the Bilaterian ancestor; they also demonstrate that despite morphological and functional differences, and more than 500 million years of phylogenic separation between *Hydra* and human, common signaling pathways are responsible for stem cell maintenance, lineage determination, and differentiation.

Keywords Epithelial stem cell, evolution of development, Hydra, interstitial stem cell, Notch, senescence, Wnt, Weismann's doctrine

Zoological Institute, Christian-Albrechts-University Kiel, Olshausenstrasse 40, 24098 Kiel, Germany
Tel.: + 49-431-880-4169, Fax: + 49-431-880-4747
E-mail: tbosch@zoologie.uni-kiel.de

T.C.G. Bosch (ed.), *Stem Cells: From Hydra to Man,*

3.1 Hydra, a Classical Model System in Developmental Biology

Cnidaria are sister group to the bilaterians (Collins, 1998; Philippe et al., 2005) and therefore provide information for reconstructing the early history of bilaterian developmental mechanisms. They consist of two epithelia, the ectoderm and the endoderm surrounding a gastric cavity; and they are the first in evolution that have a defined body plan, a nervous system, and a tissue layer construction. Cnidarians such as the freshwater polyp *Hydra* have a long history as model systems in developmental biology because of their remarkable capacity to regenerate. This ability for self-organization is at least partially due to the continuous presence of stem cells with high self-renewal capacity and high phenotypic plasticity in adult tissue. The capacity for constant renewal is also the main reason behind Hydra's potential immortality.

In molecular terms, *Hydra* as all other members of the phylum Cnidaria is astonishingly complex. The genomes in different *Hydra* species vary in size but in general are large with *H. vulgaris* having a genome of 1,250 Mbp (Zacharias et al., 2004). Moreover, Cnidaria not only have about the same number of genes as human and share most of their genes with human (Miller et al., 2005; Miller et al., 2007) but their protein sequences, surprisingly, are often more similar to mammalian sequences than to those from fly and worm (Kortschak et al., 2003). Thus, at the level of genomic complexity and gene complement, *Hydra* is much more complex than previously imagined. Novel computational tools and the development of genomic resources over the past few years have brought a new perspective on *Hydra* as a model organism. A National Science Foundation-funded large-scale *Hydra* EST Project (www. hydrabase.org) resulted in 170,000 ESTs. A National Human Genome Research Institute-funded *Hydra* genome project at the J. Craig Venter Institute currently provides 6x coverage of the *Hydra magnipapillata* genome with an assembled draft genome sequence appearing later in 2007. For database searches, Georg Hemmrich in my group has established a local Blast-platform, www.compagen.org, containing selected raw genomic (NCBI Trace archive) and EST (NCBI dbEST, JGI) sequence datasets from sponges and cnidarians up to the lower vertebrates (Hemmrich and Bosch, in prep.).

While there is no evidence that *Hydra* is simpler in molecular terms than vertebrates nor that *Hydra* cells are fundamentally different from those in mouse or human, there may be a profound difference in the differentiation potential and plasticity of the cells between *Hydra* and vertebrates. Vertebrates depend on specialized cells with limited differentiation potential to perform sophisticated functions. Cells in *Hydra*, in contrast, are capable to produce and receive positional signals continuously even in adult tissue and, therefore, have features which most cells in vertebrates have only during the short period of embryogenesis. It is this feature which makes adult *Hydra* tissue different from tissue of all other invertebrates and vertebrates.

3.2 Key Properties of Stem Cells in Hydra

Stem cells in *Hydra* represent one of the most ancient stem cell systems in the animal kingdom and, therefore, provide information for reconstructing the early history of stem cell control mechanisms. In *Hydra*, there are about 20 cell types distributed among three stem cell lineages. Each of the epithelial layers is made up of a stem cell lineage, while the remaining cells are part of the interstitial stem cell lineage which resides among the epithelial cells of both layers. Both the epithelial cells as well as the interstitial cells in the body column continuously undergo self-renewing mitotic divisions. As result of these tissue dynamics, cells in *Hydra* are constantly displaced either apically onto the head, or basally onto developing buds, or onto the foot (for recent review see Bosch, 2007a). Non-dividing differentiated cells of all three lineages are lost by displacement from the body column within 20 days (Campbell, 1967). Dividing stem cells of the interstitial lineage have a cell cycle time of 18–30 h, while stem cells of the epithelial lineages are proliferating with a doubling time of about 3.5 days (David and Campbell, 1972; Bosch and David, 1984) have a cell cycle time of 3–4 days. Hence, cells in *Hydra* are either constantly renewing by cell division, or they are lost from the animal in a relatively short period of time. An individual cell, therefore, does not exist long in a *Hydra* body.

3.2.1 Epithelial Stem Cells in Hydra: Unipotent Stem Cells with Remarkable Phenotypic Plasticity

Epithelial cells in the *Hydra* body column continuously undergo mitotic divisions (Dübel et al., 1987; reviewed in Bosch, 2007a). To prove that *Hydra* epithelial cells indeed have stem cell properties, we have made use of transgenic polyps and transplanted a single GFP-expressing endodermal epithelial cell into a nontransgenic polyp. By doing so we (Wittlieb et al., 2006) have generated polyps in which the entire ectodermal or endodermal epithelium contains the transgene. Thus, *Hydra* epithelial cells are capable, by successive divisions, both of indefinite self-renewal and of producing different types of specialised cells such as tentacle or foot specific epithelial cells. Since there is no evidence for subpopulations of epithelial cells which cannot repopulate the host tissue, all *Hydra* epithelial cells in the gastric region, therefore, must be considered as stem cells.

Supporting earlier observations, our *in vivo* tracking of GFP labelled epithelial cells also showed that there is continuous tissue displacement towards the extremities. Displacement of ectodermal epithelial cells into the tentacles results in differentiation of battery cells which contain cnidocytes. Displacement of epithelial cells towards the lower body regions results in differentiation of epithelial cells into basal disk cells which secret mucus. Other examples for epithelial cells with diverse architectural designs and physiology include the endodermal epithelial cells surrounding the gastric cavity, and the ectodermal epithelial cells encasing the

body, the tentacles, the testes, and the egg cup holding the developing oocyte. This remarkable morphological plasticity of epithelial cells in response to positional signals allows *Hydra* with only a limited number of cell types to generate structures that display a fascinating array of various cellular architectures, each of which are specifically tailored for distinct functions.

To execute these different programs of terminal differentiation, epithelial stem cells must be instructed by their microenvironment. Although the precise mediators for the different positional signals along the body column are not well defined yet, secreted Wnt ligands may be one of the first signals involved in this communication. In vertebrates, Wnt/β-catenin signalling pathways have been shown to control the specification, maintenance, and activation of epithelial stem cells (Reya and Clevers, 2005). Consistent with that, treatment of *Hydra* with alsterpaullone, which specifically blocks the activity of GSK-3β, induces rapid transformation of body column epithelial cells into an epithelial cell with morphology typical for the head region (Broun et al., 2005; Anton-Erxleben et al., in prep.). While these observations provide direct evidence for the involvement of the canonical Wnt pathway in controlling epithelial stem cell behavior, there is no clear understanding yet how β-catenin/Tcf signalling regulates epithelial stem cells in *Hydra*. Identification of the Wnt targets by transcriptional profiling might contribute to fully understand the effect and potency of β-catenin/Tcf activity in epithelial stem cells.

3.2.2 Hydra's Interstitial Stem Cells – Following the Hematopoietic Trend

Hematopoietic stem cells (HSC) and their progressively committed progeny have become the prototype examples of what might be expected of candidate stem cells in other organisms (Metcalf, 2007). Maintenance of the HSC is central to the lifelong production of blood cells by the hematopoietic system. Tests for proving the self-renewal and the plasticity of HSCs are based on injection into a mouse that has received a dose of irradiation sufficient to kill its own blood-producing cells. If the mouse recovers and all types of blood cells reappear (bearing a genetic marker from the donor animal), the transplanted cells are deemed to have included stem cells. These studies have revealed that there are two kinds of HSCs, long-term stem cells that are capable of self-renewal and short-term precursor cells capable of proliferating, but with a limited capacity to differentiate into more than one cell type. Another key feature of hematopoietic stem cells is their ability to migrate in a site-specific fashion and their interaction with their niche, a unique environment that is able to confer stem-like properties on occupying cells. The Wnt/β-catenin and Notch pathway are potent regulators of HSC function (Duncan et al., 2005; Trowbridge et al., 2006). *Hydra*'s interstitial stem cells are remarkable similar in many aspects to HSCs.

3.2.2.1 Multipotency of Interstitial Stem Cells

Interstitial stem cells are multipotent and able to differentiate into several different cell types (Fig. 3.1; Bosch, 2007b). The direct demonstration of the existence of multipotent interstitial stem cells in *Hydra* (David and Murphy, 1977) relied on the method of cloning HSCs in lethally irradiated mice (Till and McCulloch, 1961, 1963). In the original procedure (David and Murphy, 1977), *Hydra* were treated with nitrogen mustard which inactivates rapidly proliferating cells of the interstitial cell lineage and causes their elimination from the tissue. The remaining epithelial tissue was used as host for culturing added interstitial cells. Interstitial cells to be cultured were introduced into host tissue using a technique for dissociating and reaggregating *Hydra* cells (Gierer et al., 1972). In a later modification of this clonal assay (Bosch and David, 1987), elimination of host interstitial cells was achieved using a mutant strain (sf-1) which contains temperature-sensitive interstitial cells as host tissue. Temperature resistant donor cells were added in low numbers to sf-1 such that the added cells grew as clones. Subsequently host sf-1 interstitial cells were eliminated by a temperature shift. This technique made possible long-term clonal culture of *Hydra* stem cells. The results indicated that interstitial stem cells are multipotent in the sense that individual stem cells can differentiate into somatic cells as well as germ line cells (Bosch and David, 1987). The results provide no evidence for the existence of subpopulations of interstitial cells with restricted differentiation capacities (Bosch and David, 1987).

3.2.2.2 Migration of Cells of the Interstitial Cell Lineage

Similar to cells of the hematopoietic lineage, cells belonging to the *Hydra* interstitial stem cell lineage but most likely not the stem cells *per se* (see below) have an extensive capacity to migrate (Heimfeld and Bode, 1984; Fujisawa, 1989; Fujisawa et al., 1990; Teragawa and Bode, 1990, 1995). Following differentiation into nematoblasts and neuroblasts in the gastric region, these cells must traverse great distances to reach their final destination in the tentacles where most of them get incorporated in a “battery cell complex” (Bosch, 2007b). To determine the intrinsic migratory behavior and to examine whether all cells of the interstitial cell lineage display high motility or whether migration is restricted to certain subpopulations, we grafted transgenic tissue containing EGFP + interstitial cells to “naïve” tissue. Since polyps are essentially transparent, live imaging of eGFP + cells demonstrated that migration of nematoblasts and neuroblasts occurs as individual cells and never as cluster of cells (Khalturin et al., 2007). Surprisingly, migrating cells were capable of rather rapid (0.2 μm/min) motility *in vivo*. Interestingly, nearly all migrating cells could be classified as nematoblasts or neuroblasts whereas interstitial stem cells (large 1 + 2s) were mostly residing at the transplantation edge and were not actively motile. This *in vivo* observation is consistent with our previous view (Bosch and David, 1990; Fujisawa et al., 1990) that interstitial stem cells in *Hydra* in contrast to differentiating interstitial cells show little if any migratory activity.

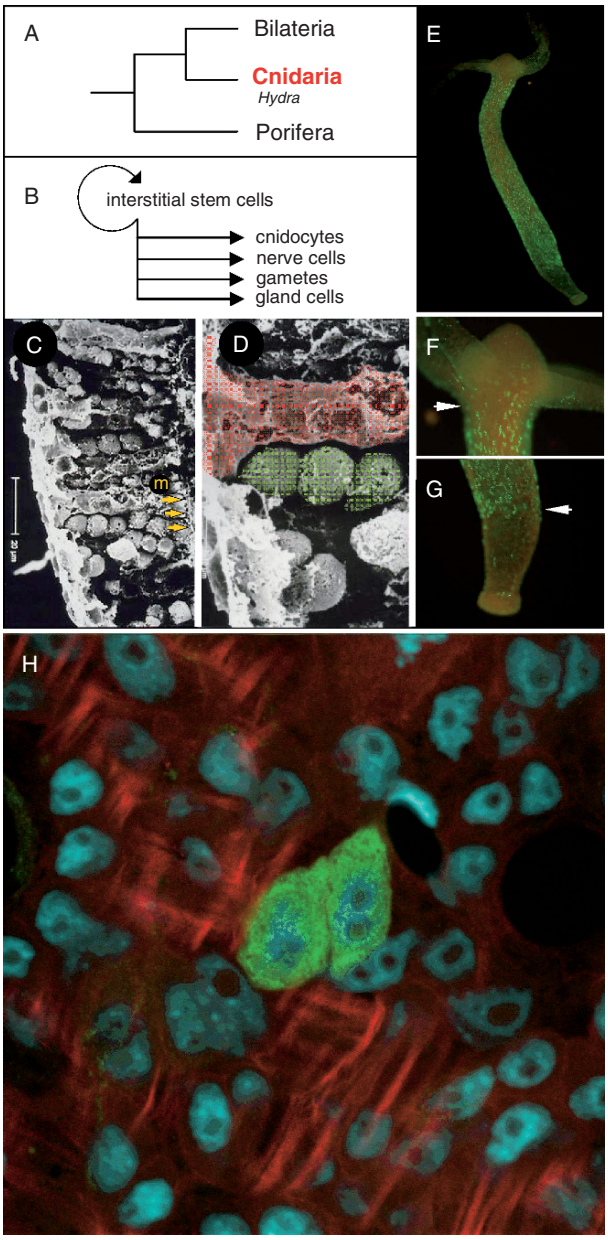

Fig. 3.1 Interstitial stem cells in *Hydra*. (A) Relationships at the base of animal evolution. Cnidaria are often regarded as the closest outgroup of the Bilateria. (B) The interstitial stem cell system in Hydra. (C) Scanning electron microscopic view of a longitudinal section through the ectoderm. m, mesoglea. Yellow arrows indicate the location of the mesoglea. (D) Scanning electron micrograph showing interstitial cells within their niche. Note the close contact of interstitial cells (green) to an ectodermal epithelial cell (red). (E) Distribution of interstitial cells in Hydra. Interstitial cells were stained with monoclonal antibody C41. (F–G) Interstitial cells (stained by monoclonal antibody C41) follow positional cues and are absent in head (F) and foot (G) tissue. Arrows indicate the border at which interstitial cells disappear. (H) A pair of transgenic interstitial cells expressing eGFP. (Modified from Khalturin et al., 2007)

3.2.2.3 The Interstitial Stem Cell Niche

Similar to the niche of HSC, *Hydra* has a distinct interstitial cell niche. Interstitial cells grow and differentiate in the interstices between ectodermal epithelial cells. To visualize their precise site of residence (referred to as niche) we performed scanning electron microscopy (Khalturin et al., 2007). Images of the ectodermal epithelium show (Fig. 3.1) chains of interstitial cells and their derivatives in close contact with the ectodermal epithelial cells. Since the mesoglea (extracellular matrix) may present an additional important acellular component of the microenvironment, the interstitial cell niche in *Hydra* appears to contain a high level of structural complexity (Khalturin et al., 2007).

3.2.2.4 The Cellular Environment Controls Interstitial Cell Behavior

Reflecting sophisticated and largely unknown signalling and growth requirements, interstitial stem cells and their derivatives display a striking position dependent distribution pattern. Interstitial stem cells are found throughout the gastric region; they are present at only very low numbers in the head and foot region (David and Plotnick, 1980). Cnidocyte differentiation occurs exclusively in the gastric region as a highly complex, multistep process (David and Challoner, 1974; David and Gierer, 1974; Shimizu and Bode, 1995; reviewed in Tardent, 1995; see also Nüchter et al., 2006). Neuron differentiation from multipotent interstitial stem cells also occurs exclusively in the gastric region (David and Gierer, 1974). After entering the neuron differentiation pathway, about a half of the neuron precursor cells migrate toward the head and foot (Heimfeld and Bode, 1984; Fujisawa, 1989; Teragawa and Bode, 1990; 1995; Technau and Holstein, 1996; Hager and David, 1997). The remaining half of the neuron precursors do not migrate, but complete differentiation and are integrated into the nerve net.

While these differentiation decisions appear to be controlled by the composition of the cellular environment, little is known about the molecular mechanisms by which the interstitial cell niche regulates interstitial stem cells. The current state of the art has been summarized recently (Bosch, 2007b). Briefly, there is accumulating evidence (Takahashi et al., 2000; Bosch and Fujisawa, 2001) that epithelial cells affect interstitial cell differentiation behavior by secreting epitheliopeptides. There is also experimental evidence that derivatives of the interstitial cell lineage such as neurons affect interstitial cell differentiation (Takahashi et al., 2000), and that nerve cell density influences interstitial cell proliferation (Bosch et al., 1991). Despite these observations, however, a direct evidence for a role of the interstitial cell niche in interstitial cell behavior and a spatio-temporal dialog between hydra interstitial stem cells and niche cells has not been demonstrated yet. Currently we use transgenesis to uncover niche characteristics by introducing alterations in the niche by gain-of-function and loss-of-function experiments. For example, does overexpression or silencing of epitheliopeptides in transgenic polyps affect nerve cell differentiation? Since it is likely that loss of neurons has broad effects on the microenvironment: what happens to interstitial cell differentiation in *Hydra* tissue

in which the number of neurons is severely reduced? Using such experimental approaches we expect to obtain direct in vivo evidence that the microenvironment plays a dominant role in interstitial cell behavior, self-renewal and multi-lineage differentiation.

3.2.3 Wnt and Notch Pathways Are Involved in Controlling Stem Cell Behavior in Hydra

To investigate the critical regulatory events that control the transformation of an interstitial stem cell into a differentiated cell, we produced transgenic polyps expressing eGFP specifically in the interstitial stem cell lineage. Because Wnt signals appear to constitute the principle driving force behind developmental processes in *Hydra* (Hobmayer et al., 2000; Broun et al., 2005), we hypothesized that alteration in Wnt signalling may specifically alter interstitial cell behavior. Wnt signalling was activated in transgenic polyps by the addition of alsterpaullone (ALP), a drug which specifically inhibits the cytoplasmic destruction complex causing degradation of β-catenin. Addition of 5 μM ALP had drastic effects on eGFP expressing interstitial cells and their derivatives (Khalturin et al., 2007). Within 48 h upon treatment, nests of differentiating nematoblasts broke up into single cells indicating terminal differentiation of all nematoblasts present in the gastric region (Khalturin et al., 2007). In untreated control polyps, nematoblast nests disaggregated progressively and new nests were produced continuously. ALP, therefore, seems to directly inhibit the differentiation of interstitial cells into nematoblasts by inducing ectopic terminal differentiation of nematoblasts in the gastric region. The expression of two genes, which are markers for nematocyte differentiation, supported this view (Khalturin et al., 2007). To determine whether the Wnt induced changes in interstitial cell behavior reflect cell-intrinsic activity or rather a response towards the changed microenvironment, we grafted tissue with eGFP + interstitial cells to unlabelled host tissue which had been treated with ALP for 48 h preceding transplantation. We observed normal migratory activity of interstitial cells and nematoblasts into ALP treated tissue indicating that ALP has no effect on the migratory activity of interstitial cells per se (Khalturin et al., 2007). In sum, extrinsic signals from the microenvironment play a major role in interstitial cell differentiation and migration, and may be mediated by the Wnt pathway. This pathway in adult Hydra obviously fulfils two functions, one in patterning (Hobmayer et al, 2000; Broun et al., 2005) and one in interstitial cell differentiation (Khalturin et al., 2007).

To further dissect the molecular mechanisms that are involved in interstitial cell differentiation, we investigated the function of the Notch pathway in vivo. We treated polyps for 48–96 h with DAPT which blocks Notch activity by inhibiting the γ-secretase-dependent cleavage that releases the Notch intracellular domain (Geling et al., 2002; James et al., 2004). A block in Notch signalling prevented nematoblast differentiation (Khalturin et al., 2007). Strikingly, nerve cell differentiation appeared

not to be affected at all. To analyze the inhibition of Notch signalling on interstitial cell differentiation at the molecular level, we studied the expression of several genes expressed in interstitial cells after entering either the nematoblast or the neuron differentiation pathway. We obtained evidence (Khalturin et al., 2007) that Notch activity is required by differentiated nematocytes, not just by the dividing cells of the nematoblast nests. Since no differences could be observed in control and treated Hydra tissue with regard to the number of neurons present, there seems to be no trade-off between nematoblast and nerve cell differentiation when Notch activity is blocked. Suppression of Notch signalling causes immediate death of differentiating nematoblasts. Inhibition of Notch does not affect neurons nor interstitial cells as the nematocyte population quickly recovers after termination of DAPT treatment. Notch, therefore, appears to be a permissive cue in nematoblast differentiation, rather than an instructive one. These observations supported a previous study (Käsbauer et al., 2007) and strongly implicate Notch signalling as a key component in the acquisition of nematocyte fate. They also point to Notch as an ancient molecular "switch" used already in early branching metazoans to distinguish different cell types. Taken together, since Wnt and Notch pathways are involved in controlling stem cell behavior in hydra, similar key signalling pathways appear to orchestrate stem cell behaviour throughout the animal kingdom from *Hydra* to man.

3.2.4 Hydra Stem Cells and the Evolution of Senescence

Senescence is generally defined as progressive declines of physiological functions, leading to an increase in the mortality rate as a function of time. Senescence has been found in all metazoans where careful studies have been carried out. Although it is often postulated that stem and progenitor cell depletion or dysfunction might contribute to senescence, the biochemical basis behind remained elusive. It came as a surprise, therefore, that Wnt proteins in stem cells were recently shown to play an instructive role in controlling the onset of senescence (Liu et al., 2007; Brack et al., 2007). Constitutive Wnt stimulation appears to contribute to stem cell depletion and aging as mice lacking a Wnt antagonist have a shortened life span and exhibit a number of age-related changes (Liu et al., 2007).

Hydra has been suggested not to undergo senescence, and being biologically immortal (Martinez, 1998; 2002). This potential immortality of Hydra has been a hotly disputed controversy over the last few decades. However, a careful and elegant study (Martinez, 1998) analyzing the mortality patterns and reproductive rates of four groups of individuals of *Hydra vulgaris* for a period of 4 years (Martinez, 1998) could find no evidence for aging in *Hydra* and no apparent signs of decline in reproductive rates. Extremely low mortality rates and lack of senescence appears to be due to the tissue dynamics and the fact that the body can be constantly renewed from populations of stem cells.

This view has been challenged recently by Yoshida et al (2006) who searched for signs of aging in sexually differentiated *Hydra oligactis*. Since after sexual reproduction Yoshida et al. (2006) found a significant decline in the capacities for food capture, contractile movements, and reproduction as well as an exponential increase in the mortality rate of the population, they proposed that the degenerative process in *H. oligactis* following sexual reproduction represents the aging process. These data, however, have to be taken *cum grano salis* for two reasons. First, these degeneration processes are observed only under laboratory conditions and might be simply the consequence of the excessive sexual production activity described by the late Pierre Tardent (1974): "Particularly in males gamete production is so intense that we can speak in terms of a "gametic crisis" ("crise gametique," Brien, 1966) leading to a complete exhaustion and death of the animals." Second, there are several species of closely related *Hydra*, e.g. *H. magnipapillata* and *H. vulgaris*, which do not undergo degeneration after sexual reproduction but continue to proliferate and grow indefinitely. Since it seems unlikely that there are immortal *Hydra* species and mortal ones, degeneration in *H. oligactis* might simply be based on the fact that in this species environmental signals cause multipotent stem cells to shift their differentiation program exclusively to germ cell differentiation.

Taken together, there is no convincing evidence for senescence in *Hydra* at the cellular and individual level. Intriguingly, this is also supported by recent observations at the molecular level in transgenic polyps with constitutive Wnt signaling in the epithelial stem cell lineage (Hartig et al., in prep). Transgenic *Hydra* that express β-catenin driven by the actin promoter have a characteristic phenotype with multiple axes as well as ectopic tentacles, supporting the view that the canonical Wnt pathway is involved in the activity of the head organizer (Hartig et al., in prep). These animals, however, continuously produce buds that subsequently develop into multiple axes animals. Epithelial stem cells in these animals, therefore, must continuously proliferate. Thus, constitutive Wnt signaling at least within the epithelial stem cell lineage in *Hydra* does not appear to lead to a rapid exhaustion of long-term repopulating stem cells. We might conclude, therefore, that the molecular language involved in control of senescence in vertebrates with the instructive role of Wnt in aging does not to work in immortal *Hydra*.

Hydra has chosen a life cycle in which proliferation occurs mostly asexual by budding. That requires that each bud obtains the complete cellular repertoire from the mother polyp. By giving all the epithelial cells in the budding region stem cell properties and by filling the interstitial space with multipotent interstitial stem cells with the potential to differentiate not only into somatic cells but also into gametes, buds obtain all what they need. Thus, it is the stem cellness of the tissue which allows *Hydra* its unique life cycle. It seems that this feature alone is sufficient to explain *Hydra*'s immortality. While young Achilles was dipped in the pool of immortality by his mother, natural selection has removed senescence from the life cycle of *Hydra*.

3.2.5 *Hydra Stem Cells Violate Weismann's Doctrine of the Continuity of the Germ Plasm*

About a century ago August Weismann observed that in hydrozoans germ cells are derivatives of "common embryonic tissue cells" found in a given part ("Keimstätte") of the tissue (1883). Based on this observation he concluded that only certain groups of predetermined cells can differentiate gametes and published his doctrine of "the continuity of the germ-line" (Weismann, 1892). Soon thereafter the idea of a complete separation in metazoans between an immortal germ line and a soma that would serve to transfer the germ products to the next generation and then senesce became very popular. In cnidarians, however, the existence of a germ line, despite Weismann's observation, has never been demonstrated experimentally. In contrast, clonal assays have shown (Bosch and David, 1987) that *Hydra* contain multipotent interstitial stem cells continuously capable of differentiating somatic as well as germ line cells suggesting a common origin of germ cells and somatic cells. No evidence was found for the existence of a particular group of cells with germ line-restricted differentiation capacities (Bosch and David, 1987). These results provide experimental support for a proposal made by Buss and Green (1985; see also Buss, 1987) that asexual proliferation by budding ("ramet production") requires the presence of an actively dividing multipotent cell line capable of somatic as well as germ line differentiation. For this reason, colonial organisms such as many cnidarians are expected to differentiate germ cells from a pool of multipotent stem cells. Comparative data from *Hydra*, flatworms and the annelid *Platynereis* (Rebscher et al., 2007; and references herein) indicate that a two-step mode in germ cell specification may be ancestral for metazoan germ line segregation.

The dual potential of interstitial stem cells is also reflected at the molecular level since multipotent stem cells as well as germ line cells both express *nanos (Cnnos)* and *vasa (Cnvas)* homologous genes (Mochizuki et al., 2000; Mochizuki et al., 2001). *Vasa* encodes an ATP-dependent RNA helicase belonging to the DEAD box protein family and is one of the most reliable markers for germline cells (Raz, 2000) throughout the animal kingdom. In *Hydra magnipapillata*, Cnvas1 and Cnvas2 both are expressed in multipotent stem cells as well as male and female germline cells (Mochizuki et al., 2001). A *Nanos* gene, encoding an RNA binding protein, was first identified as a maternal effect gene in *Drosophila* (Wang and Lehmann, 1991). To date, *nanos*-related genes have been cloned in several invertebrates and vertebrates and found to play a critical role in germ cell development. In *H. magnipapillata*, Cnnos1 is expressed in both multipotent stem cells and germ line cells while Cnnos 2 shows a less clear expression pattern (Mochizuki et al., 2000). Although the expression pattern of Cnos1 and Cnvas1 is suggestive, a function of these proteins in regulating germ cells has not been demonstrated yet.

In sum, as stated 2 decades ago (Bosch and David, 1987), *Hydra*'s pattern of germ cell development characterized by the absence of early terminal differentiation clearly violates Weismann's doctrine. Weisman held that there existed a "molecular

distinction" between the germ plasm and the soma, such that the soma was merely a mortal vessel upon which selection acts. Phylogenetic distribution of this trait, however, shows that early terminal differentiation is a character limited exclusively to some higher metazoan taxa. In *Hydra* and other early-branching metazoans, cells destined to become gametes derive from multipotent stem cells which display a dual potency. Unravelling how *Hydra* interstitial stem cells become determined to differentiate into gametes, and how the germ cells are specified remains one of the great challenges for the future.

3.2.6 Continuous Self-Renewal and the Risk of Developing Cancer

The evolution of multicellularity necessitated the development of strict controls to keep cellular proliferation in check. Stem cells possess an enormous developmental potential and have the unique ability to self-renew. These two features essential for their normal behaviour could make stem cells a major threat to the organism if the machinery that keeps them in check becomes defective. One interesting thought (Clarke and Fuller, 2006) is that long-lived organisms have developed a strategy to limit the number of long-lived cells with self-renewal capacity. Restricted long-term renewal of short-lived cell types may reduce the chance that a single cell with proliferative capacity will accumulate the mutations required for malignant transformation.

As described above, in *Hydra* every epithelial cell in the body column has stem cell feature and stem cells of the interstitial cell lineage are abundant throughout the tissue and continuously undergo self-renewing mitotic divisions. Despite this potential vulnerability to cancers, *Hydra* tissue, however, never shows signs of malignant transformation. Although up to now the regulatory mechanisms involved remain completely elusive, a plausible reason for the absence of malignant cells which have loosened themselves from the constraints on proliferation may be that all cells in *Hydra* including the stem cells are short-lived cells which rapidly get displaced towards the ends of the body axis due to the tissue dynamics. Thus, the chance that a single *Hydra* cell will accumulate the mutations required for malignant transformation is greatly reduced. While higher organisms may have reduced the number of continuously dividing cells and evolved small populations of stem cells as a protection against cancer (Clarke and Fuller, 2006), in *Hydra* there simply may be no need to limit the number of stem cells since due to the tissue dynamics all cells are short-lived. Another aspect contributing to the conspicuous absence of cancer in long-lived cnidarians may be their extensive repertoire of metabolic signalling (Blackstone, 2006) and the fact that redox signalling could activate signalling pathways involved in balancing cellular proliferation and quiescence (Blackstone, 2007).

Hydra tissue, however, is not completely free of the risk of hyperproliferation and developing tumors. In a culture of *H. oligactis*, a polyp with drastic alterations in the tissue composition was identified (Anokhin et al. in prep). Loss of anatomical

integrity of the ectodermal and endodermal epithelium resulted in tumor-formation and loss of capacity to bud. Longitudinal sectioning followed by regeneration allowed the production of a clonal culture of polyps which all displayed the same tumor-bearing phenotype. To get first insight in the cellular and molecular background for this phenotype, tumor-bearing polyps were analyzed using a variety of histological and molecular techniques. Since microscopic examination revealed large, pale multilobulated masses composed of interstitial cells, loss of tissue integrity appears to result from loss of cellular homeostasis and increased interstitial cell proliferation. In situ hybridization using the germ cell specific gene *nanos* as probe showed that the rapidly proliferating interstitial cells were primordial germ cells. Thus, these Hydra tumors have histologic and molecular features of ovarian germ cell tumors (dysgerminomas) as described in mammals. While germinal cell tumours represent 2–5% of all cancers of the ovary, ovarian tumors are uncommonly encountered outside mammals, and never observed so far in any invertebrate. This observation, therefore, is the first report of ovarian germ cell tumor in any early-branching metazoan species. Identifying the biochemical basis of tumor formation will undoubtedly contribute to our understanding of the mechanisms that normally control tissue homeostasis and cell proliferation in *Hydra*; and that allow these potentially immortal organisms to simultaneously escape both cancer and senescence. Moreover, as molecules and pathways involved in biological processes are evolutionary conserved and most vertebrate gene families have deep evolutionary roots (Kortschak et al., 2003, Miller et al., 2005; Technau et al., 2005), greater understanding of how germ cell differentiation is regulated in *Hydra* could also have clinical significance. Since the cause of ovarian cancer is unknown, *Hydra*'s position at the base of animal evolution might be advantageous and useful for the identification of genes controlling key steps in germ cell differentiation.

3.2.7 Stem Cells Are Not Enough to Keep Up with the Tissue Dynamics: Gland Cell Complexity in Hydra Is Maintained by Both Stem Cell Based Mechanisms and Transdifferentiation

Three of the interstitial cell differentiation products are gland cells found in the endoderm of the body column, and two types of mucous cells found in the endoderm of the hypostome, the apical part of the head. Little was known until recently about gland cell differentiation from interstitial stem cells (Schmidt and David, 1986; Bode et al., 1987). It was assumed but was not shown yet that the mucous cells in the head are also derived from the interstitial cells. We re-addressed the issue of gland cell differentiation by generating transgenic *Hydra* in which eGFP expression was under control of the promoter of a gland cell specific gene, HyDkk1/2/4 C (Siebert et al., 2008). The fact that transgenic *Hydra* recapitulated faithfully the previously described graded activation of HyDkk1/2/4 C expression along the body

column, indicated that the 1.027 bp promoter contains all elements essential for spatial and temporal control mechanisms. Tracing individually labelled zymogen gland cells (ZMGs) allowed us to show that continuous changes in position along the single body axis are accompanied by continuous changes in gene expression and morphology; and to define a distinct ancestor-descendant relationship between ZMGs in the gastric region and granular mucous gland cells (gMGC) in the head. In addition we observed that part of the mucous gland cell population in the head is directly derived from interstitial cells. The *in vivo* observations, therefore, show that in *Hydra* both stem cell-based mechanisms and transdifferentiation are required for maintaining a population of differentiated cells in the context of active tissue dynamics. The results demonstrate a remarkable plasticity in the differentiation capacity of cells in an organism which diverged before the origin of bilaterian animals. The studies reveal differentiation in *Hydra* to be a surprisingly dynamic process and, to our knowledge, provide the strongest evidence to date that transdifferentiation *in vivo* plays a major role in maintaining cell complexity.

3.2.8 Epigenetic Control of Interstitial Stem Cell Differentiation

Epigenetic genome modifications are important for specifying pluripotency and lineage commitment (Azuara et al., 2006; Spivakov and Fisher, 2007). Studies examining specific epigenetic features of human and mouse stem cells – such as the abundance of modified histones, Polycomb group (PcG) protein-binding patterns, and chromatin accessibility – have provided important insights into the unique properties of pluripotent stem cells. PcG proteins form multiple Polycomb Repressive Complexes (PRCs) and are epigenetic chromatin modifiers involved in maintenance of embryonic and adult stem cells (Plath et al., 2003; Valk-Lingbeek et al., 2004). The PRC2 complex, comprising embryonic ectoderm development (EED), Enhancer of zeste (EZH2), and additional components, initiates gene silencing and catalyzes histone H3 methylation on lysine 27 (H3K27) at target loci (Kirmizis et al., 2004; Kuzmichev et al., 2004). Embryonic stem cells appear to manage their pluripotent status by "keying up" important regulator genes for future expression, using a PcG-mediated repressive histone lock (Spivakov and Fisher, 2007). This prevents precocious expression of genes that drive the differentiation along specific differentiation pathways, but also allows the same genes to be primed for future expression. Thus, the default state appears to be "differentiation" while "stemness" must be actively maintained.

We previously have shown that in *Hydra* the gene encoding Polycomb protein HyEED is specifically expressed in interstitial cells and differentiating nematoblasts (Genikhovich et al., 2006). HyEED is not expressed at later stages of differentiation and, therefore, absent in the head and foot regions. In male polyps, HyEED is co-expressed with the *Hydra* homologue of EZH2 (Genikhovich et al., 2006). Since sperm precursors expressing HyEED show high levels of histone methylation (Genikhovich et al., 2006) and co-express HyEZH2, the PRC2

complex appears to be involved in remodeling and silencing sperm chromatin. To explore whether epigenetic histone modifications are important for differentiation of interstitial cells, we produced polyps which overexpress HyEED in the interstitial cell lineage under the control of the *Hydra* actin promoter (Khalturin et al., 2007). Unexpectedly, the localization of the fusion protein emulates the endogenous expression of HyEED. Confocal microscopy showed HyEED-eGFP expression in interstitial cells as well as developing nematoblasts. Other derivatives of the interstitial stem cells were not observed to express the fusion protein.

To examine whether the disappearance of the eGFP signal in the head and foot tissue is correlated with terminal differentiation, we monitored HyEED-eGFP positive cells located in the gastric region as well as during regeneration. HyEED-eGFP is actively degraded during the transition of a nematoblast into a mature nematocyte. Inhibition of the proteasome system by the MG132 membrane-permeable proteasome inhibitor (Nencioni et al., 2006) leads to presence of HyEED-eGFP + nematocytes in head and tentacle tissue whereas in control polyps such transgenic cells are never observed in these regions. Thus, the ubiquitin proteasome system is involved. We have proposed (Khalturin et al., 2007) that nematoblasts entering head or foot territory (the region of terminal differentiation) abruptly loose the HyEED-eGFP fusion protein by proteolytic degradation to facilitate terminal differentiation. Our overexpression construct seems to be unable to override this endogeneous control mechanism.

Taken together, the observations support the view that remodelling of chromatin structure is involved in interstitial cell differentiation and that – similar to cells in higher animals – HyEED is likely to actively suppress final differentiation steps in *Hydra* interstitial stem cells. Determining how these epigenetic features relate to the transcriptional signatures of stem cells, and whether they are also important in other types of stem cells in *Hydra*, is a key challenge for the future.

3.3 Problems and Prospects

Although substantial efforts have been made in recent years to identify the molecular regulators of stem cells in *Hydra*, we have as yet gained only a rough outline of the interplay of mechanisms that coordinate proliferation and differentiation of the three stem cell lineages. Figure 3.2 illustrates the current view of the differentiation and proliferation potential of interstitial stem cells in *Hydra*. Despite this progress, the information yielded from current studies only paints part of the picture, and many challenges for the future remain.

Most importantly, the fundamental question “What are the signals that control stem-cell proliferation and dictate whether a daughter of a stem cell shall remain a stem cell or become committed to differentiation?” is still unanswered. The control of stem cell numbers, their commitment, and progeny generation are biological questions of great importance in *Hydra* as in any other organism.

Much remains also to be learned about the transcription factors that regulate stem cell behavior in *Hydra*. Wnt signalling, for example, ultimately controls

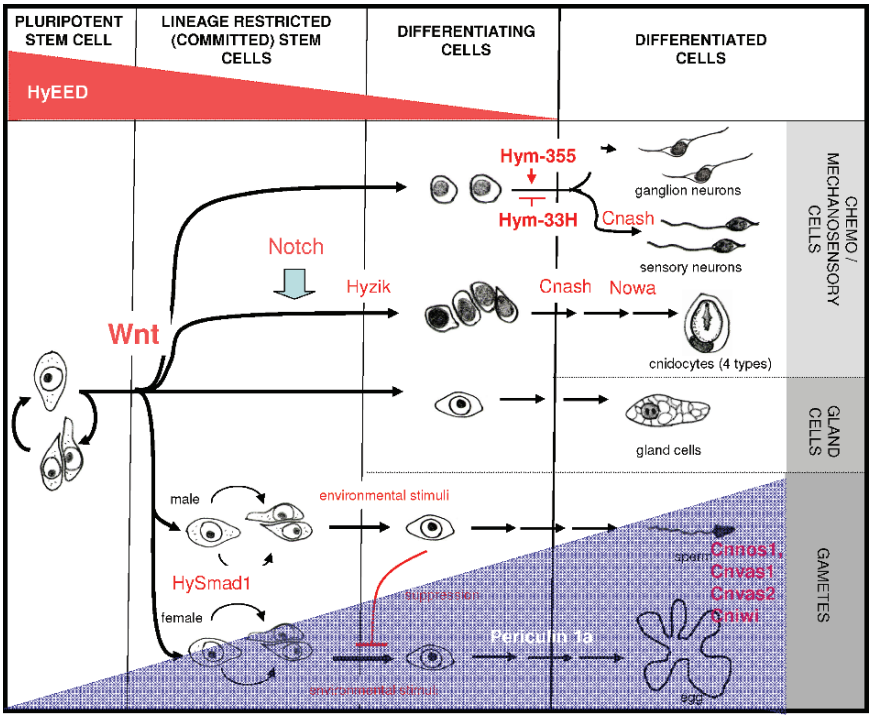

Fig. 3.2 A model for the differentiation of interstitial stem cells in Hydra. Factors affecting steps of differentiation are shown in red. For details and references see text. (Modified from Bosch, 2007b)

developmental fates through the transcription of cell-type specific programs of Tcf target genes. The gaps in our knowledge are due to the fact that conserved stem cell specific transcription factors such as OCT4, REX11, SOX2, and Nanog all are missing in the *Hydra* genome. We, therefore, must discover which genes lie upstream and are in control of pluripotency and self-renewal capacity. With advances in cell sorting and array-based technologies, we expect that the dissection of the gene programs in *Hydra* stem cells before and after differentiation decisions will provide a wealth of insight into the biology of these processes. At the end, these efforts should put one of the grails of stem cell biology in sight – reconstruction of the gene set controlling stem cell behavior in the common ancestor.

Furthermore, in *Hydra* three independent stem cell-lineages co-exist: (i) ecto – and (ii) endodermal epithelial stem cells as well as (iii) interstitial stem cells. In the long run, *Hydra*, therefore, appears to be an excellent model for studying how the divisions of three distinct types of stem cells within an organism are controlled – a question central to the understanding of tissue and cell type homeostasis.

Little is known about the epithelial stem cells in *Hydra*. As I have outlined elsewhere (Bosch, 2007a), epithelial stem cells in *Hydra* show features which can also be found in intestinal stem cells in the vertebrate gut (Bosch, 2007a). How

these epithelial stem cell are maintained, and which environmental components endow stem cell properties to them, are intriguing questions. Moreover, what features do epithelial stem cells and interstitial stem cells share? As characteristics of both epithelial and interstitial stem cells emerge, it will be interesting to ascertain what features they will share that might account for their common properties of self-renewal and repression of differentiation state. It will also be exciting to uncover the features that uniquely define them, such as their unipotency versus multipotency?

Resolving the function of the evolutionarily conserved PIWI/Argonaute family of proteins also seems fundamentally important in understanding of the intrinsic cellular processes serving as determinants of asymmetric-segregating cell fates (Bosch, 2004; Peters and Meister, 2007; Seto et al., 2007). Members of this protein family are found in plants, yeast, and throughout the animal kingdom; and define the first family of evolutionarily conserved genes that are essential for stem cell division in both animal and plant kingdoms.

Lastly, *Hydra* is the only cnidarian in which multipotent interstitial stem cells have been found so far and in which it was clearly shown that germ cells continuously differentiate from multipotent stem cells. The origin of germ cells in well-studied anthozoans such as corals or *Nematostella*, but also in scyphozoans such as *Aurelia* or other hydrozoans (*Podocoryne*) remains enigmatic. Where do they come from? Do these cnidarians lack the interstitial cell lineage and produce all their cell types from only two stem cell lineages, the ectodermal and endodermal epithelial cells? Does, therefore, development of the interstitial cell lineage in *Hydra* reflect an evolutionary relatively young event by which the population of endodermal epitheliomuscular cells segregated into a unipotent endodermal epithelial and a multipotent interstitial cell lineage?

Despite these problems and open questions, the imminent availability of methods for functional analyses of cnidarian genes and the massive advances in molecular technology that are presently taking place, make *Hydra* a powerful and also intellectually attractive system for studying stem cells since it allows easy access to combined genetic, cell biological, molecular and computational approaches. In particular, transgenic *Hydra* are paving the way for many applications including in vivo imaging to analyze stem cell behavior and niche function in an animal that diverged from the main line of metazoan evolution about 560 million years. Thus, since fundamental processes that are relevant for understanding asymmetric division and self-renewal are expected to be conserved in the animal kingdom, with the molecular dissectioning of the components controlling epithelial homeostasis and decision making in *Hydra*, the stage is set for lower metazoan biologists to uncover the mystery of "stemness" and deciphering the fundamental components controlling pluripotency and lineage commitment that underlie all stem cell systems. There is still a lot to learn.

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Chapter 4
Stem Cells in Planarian

Kiyokazu Agata

Abstract The high regenerative power of planarian has fascinated scientists for a long time. Although many famous scientists, including Thomas Hunt Morgan, extensively devoted themselves to studying mechanisms underlying regeneration, the planarian regeneration still remains mysterious phenomenon. Recently, modern approaches have been developed and incorporated in planarian research, and several mysterious points were clarified. Here, I focus on old problems of planarian stem cells. Planarian stem cells were called "neoblasts" and considered to be the only cells maintaining proliferative activity and pluripotency. However, recent analyses using molecular markers and FACS sorting revealed their heterogeneity. Now we should change the old view of the planarian stem cell system to one closer to mammalian stem cell systems. These insights indicate the planarian regeneration studies may provide new ideas for handling mouse and human ES cells for therapeutic use in the near future.

Keywords planarian, regeneration, neoblast, stem cell, pluripotent, totipotent, FACS

4.1 Introduction

It is well known that planarians have strong regenerative ability (Fig. 4.1), and it has been believed for a long time that this ability is supported by pluripotent stem cells, called "neoblasts" (Baguñà, 1981; Baguñà et al., 1990). However, nobody has succeeded yet in demonstrating the pluripotency of the planarian neoblasts in a scientific way so far. To demonstrate the pluripotency of the cells, it will be indispensable to develop clonal cell culture, single cell transplantation techniques or GFP labeling by stable gene transformation (Osawa et al., 1996). In the case of

Department of Biophysics, Graduate School of Science, Kyoto University,
Kitashirakawa-Oiwake, Sakyo-ku,, Kyoto 606-8502, Japan
Tel.: 81-75-753-4200, Fax: 81-75-753-4203
E-mail: agata@mdb.biophys.kyoto-u.ac.jp

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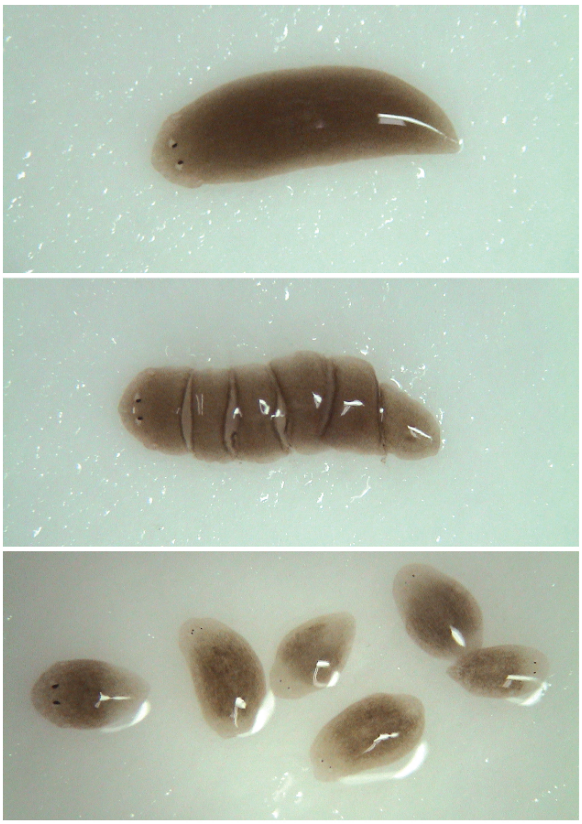

Fig. 4.1 Planarian shows high regenerative ability utilizing the pluripotent stem cell system (upper panel: before amputation, middle panel: just after amputation, lower panel: one week after amputation)

mouse, the differentiation potentiality of each type of stem cell has been demonstrated by using a variety of techniques, such as clonal cell culture, colony forming assays, rescue experiments by transplantation of single stem cells into X-ray-irradiated hosts and chimeric mouse production (Bradley et al., 1984). In this chapter we review the recent progress of studies on planarian stem cells at the cellular and molecular levels. These recent analyses have revealed heterogeneity of planarian stem cells, resulting in a different view from the classical one.

Before we enter into the main text, several important properties of planarians and some key terminology should be explained. In addition to being able to regenerate amputated or lost body parts, planarians also normally maintain their body proportion by tissue homeostasis. Thus, old differentiated cells may be constantly replaced by freshly differentiated cells derived from the stem cells (Baguñà, 1981). If one feeds a sufficient amount of food to planarians, the ratio of freshly differentiated cells to lost cells becomes greater than one, resulting in proportional growth. On the

other hand, if one starves planarians for a long time, the ratio becomes less than one, and the planarians proportionally degrow. That is, the planarian stem cell system is not activated only after amputation. It works constantly and contributes to cell renewal/tissue homeostasis as well as regeneration.

Another important preliminary point is terminology. In the past, the neoblasts were called "totipotent" stem cells, but recently it has become clear that we must instead call them "pluripotent" stem cells (National Institutes of Health 2001). In the classical definition, cells that can give rise to all cell types including germ cells were called "totipotent" cells, while cells lacking the capability to differentiate into germ cells although they can differentiate into any type of somatic cell, were called "pluripotent" cells. This distinction was made because among cells of multicellular organisms, cells maintaining the capacity to produce the next generation of offspring should have a premier position. Thus, the planarian neoblasts, which may differentiate to germ cells during the course of sexualization (Kobayashi et al., 2002), were called "totipotent" stem cells. However, the definition of "totipotent" was revised in 2001 after the establishment of human embryonic stem (ES) cells. Initially, ES cells were called "totipotent" stem cells although nobody had demonstrated the capability of human ES cells to differentiate to germ cells. However, if we called human ES cells "totipotent" cells, nonspecialists might imagine that humans could be born from cultured human ES cells. For this reason, it was decided to call ES cells "pluripotent" cells. Now, "totipotent" is used only to designate fertilized eggs, which can develop into adult organisms in a cell-autonomous way. Accordingly, we should change to calling planarian neoblasts "pluripotent" rather than "totipotent" stem cells.

The other problem of terminology is the use of the word "neoblasts". We are still using the word "neoblasts", although recent molecular and cellular studies have clearly indicated their heterogeneity (Sato et al., 2006; Higuchi et al., 2007). We should reconsider what "neoblasts" are and re-define the names of planarian stem cells. However, now is still in an intermediate period of discovery in this field, and we need more time to define the cells involved in regeneration. So, here I use "neoblasts" sometimes, and "stem cells" at other times. When I would like to refer to a group of cells showing undifferentiated cell morphology, I used "neoblasts".

4.2 History of Planarian Stem Cell Research

The neoblasts were first christened by Randolph (1897) and again by Dubois (1949). Randolph reported that spindle-shaped strongly basophilic cells could be followed during regeneration migrating to the wound and building the regenerating blastema. Dubois found that these cells were sensitive to X-ray–irradiation, and X-ray-irradiated planarians lost regeneration capability. At the end of the 19th century, Thomas Hunt Morgan demonstrated that a piece corresponding to 1/279th of a

planarian could successfully regenerate an entire planarian body (Morgan, 1898). This finding convinced scientists that pluripotent stem cells exist throughout the entire planarian body. Here, I summarize key past findings regarding the pluripotency of planarian stem cells.

4.2.1 Cytological Observations

Pederson (1959) observed the planarian neoblasts cytologically, and also using the electron microscope (EM). Morphologically, neoblasts share many attributes with the undifferentiated cells of other organisms, such as scant cytoplasm, and a large nucleus with extensively decondensed DNA. Interestingly, Wetzel (1961) also described "resting" neoblasts not engaged in migrating to the wound or in the process of differentiation in the blastema; he described them as roundish shaped, with an ovoid nucleus containing one to three nucleoli. Although several old publications had described some morphologically different neoblasts, the neoblasts have generally been looked on as a homogeneous population. However, in the 1990s Hori published several EM observation studies suggesting that some cells migrating to the wound to participate in regeneration show slightly differentiated features, such as endoplasmic reticulum formation and so on (Hori, 1992, 1997). Thus, he proposed calling these cells "regenerative cells" as distinguished from the neoblasts. He supposed that these cells may have become committed to differentiation pathways.

Another remarkable feature of neoblasts is the presence of an electron-dense, membrane-lacking chromatoid body in their cytoplasm (Hay and Coward, 1975; Hori, 1982). A close relationship is usually observed between chromatoid bodies and pore regions in the nuclear envelope in regenerative cells (Hori, 1982; Auladell et al., 1993). The chromatoid bodies continue to decrease in size during the cytodifferentiation of regenerating cells, though they do not disappear entirely during the regenerative process (Hori, 1982, 1997). It has been reported that following regeneration, chromatoid bodies remain only in germline cells (Teshirogi, 1962; Teshirogi and Ishida, 1987). The presence of RNA in the chromatoid body has been shown by cytochemical studies (Hori, 1982) and RNase treatment experiments (Auladell et al., 1993), suggesting that the chromatoid body may be a huge RNA-protein complex.

4.2.2 X-Ray Irradiation

Classic studies of Wolff and Dubois provided the most important indication of the role of neoblasts in planarian regeneration (Wolff and Dubois, 1948). They demonstrated that the ability of planarians to regenerate was lost upon X-ray irradiation. They showed that neoblasts, the only proliferating cells in the animal, are particularly sensitive to irradiation. After loss of the neoblasts, the planarian loses its ability both to regenerate

and to renew its tissues. Wolff and Dubois also examined the regenerative ability of irradiated planarians after shielding various portions of the body, and thereby found that the length of time required for regeneration is proportional to the distance between the cut edge of the body and the front of the non-irradiated region. This was the first experiment suggesting the migration of neoblasts from the unirradiated tissue to the irradiated portion of the body.

4.2.3 Transplantation

In the 1980s, the group of Baguñà and Saló conducted several transplantation experiments to investigate the properties of the neoblasts (Baguñà et al., 1989). They tried to concentrate the neoblasts using the Ficoll density gradient method after filter filtration, and then transplanted them into X-ray-irradiated planarians. By transplanting a mixture consisting of heterogeneous cell populations, they succeeded in rescue of X-irradiated hosts. Baguñà concluded by these experiments that neoblasts are "totipotent" stem cells. They also tried to follow the movement of neoblasts and differentiated cells, and showed that this migration of neoblasts is not active migration toward the blastema, but rather cell spreading due to proliferation. Faster rates of cell movement were shown to be correlated with higher mitotic indices, and cells did not appear to be driven preferentially toward the wound area.

4.2.4 Cell Culture

Although many researchers have tried cell culture of the planarian neoblasts, nobody has succeeded in obtaining their proliferation under in vitro conditions (Teshirogi and Ishida, 1987). One of the reasons is that the contaminating intestinal cells or fragmented cells release a lot of proteases and toxic materials, killing neoblasts. To demonstrate pluripotency of the neoblasts, establishment of a cell culture system will be indispensable.

4.3 Recent Progress of Planarian Stem Cell Research: (I) Cellular Level

Although many people have tried to investigate the planarian stem cell system, current methods lack the resolution to distinguish between pluripotent stem cells and their descendants committed to particular lineages. However, recently, many new methods have been developed, and new knowledge has been accumulated. Here, I summarize recent analyses and progress at the cellular and molecular levels achieved using modern techniques.

4.3.1 Detection and Visualization of Dividing Cells

BrdU incorporation experiments are indispensable not only for detecting proliferating cells but also for tracing the fate of the stem cells after division. However, for a long time, nobody succeeded in incorporation of BrdU into planarians using standard methods, and this was a stumbling block in planarian stem cell research. Newmark and Sánchez Alvarado overcome this problem in 2000 by feeding planarians BrdU along with their food (Newmark and Sánchez Alvarado, 2000). This made it possible to detect the proliferating cells under whole-mount conditions and trace the fate of the stem cells. They demonstrated the restricted distribution of the dividing cells in the body. No proliferating cells were detected in the head region or in tissues composed of differentiated cells. BrdU-incorporating cells were uniformly distributed throughout the mesenchymal space of the body except for the head region. However, within several days after BrdU labeling, many labeled cells were observed in the head region and differentiated tissues, suggesting that the stem cells in the mesenchymal space of the trunk region actively migrated into the head region and participated in tissue homeostasis. This was an important study demonstrating that in planarian, actively proliferating stem cells are not distributed in the head region. This may be a key point for understanding the planarian stem cell system. They also showed that drastic elevation of the proliferation of the stem cells after amputation was not observed. Accordingly, they revised the G2-arrest theory of planarian stem cells in this paper, concluding that planarian stem cells constantly divide not only to maintain homeostasis but also to contribute to regeneration, without G2-arrest.

Sánchez Alvarado and Orii also succeeded in detecting M- and S-phase cells using anti-phosphorylated histone H3 and anti-PCNA antibodies, respectively (Newmark and Sánchez Alvarado, 2000; Ito et al., 2001; Orii et al., 2005). In the case of PCNA, commercially available antibody does not cross react with the antigen of planarians. Orii isolated the PCNA gene using a degenerate PCR method, produced its protein in bacteria and then raised antibodies by injection of the fusion protein into mouse. Combined immunostaining with these antibodies and BrdU incorporation experiments confirmed some of the classical observations, but also led to some revision of the classical concept.

4.3.2 Tracing of Cells Participating in Regeneration by Chimeric Analysis

To trace cell fate during regeneration, chimeric analysis is one of the most powerful methods. Studies of chicken-quail chimeras are well known to contribute to the tracing of neural crest cells. To achieve a similar purpose, we extensively searched for probes recognizing cells derived from different species and strains. We found one retrotransposon, named PH20, which was specifically expressed in our laboratory strain of *Dugesia japonica* (GI). However, transplantation among different species

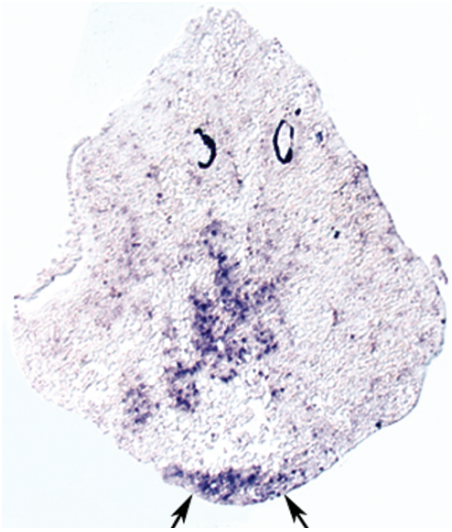

Fig. 4.2 Contribution of committed stem cells to blastema formation demonstrated by chimeric analysis, After plantation of GI tissues into the head region of the HI host, the chimeric planarian was amputated posterior to the head, and the contribution of donor cells (stained in blue using PH20 RNA probe) to blastema formation (indicated by arrows) 3 days after amputation was observed

did not work well. Thus, we carried out transplantation between members of the same species but different strains. We were breeding two different laboratory strains (GI and HI) of *Dugesia japonica*, and we could distinguish the cells of these two strains using the PH20 probe, since GI cells are much more strongly stained with the PH20 probe, even though both strains carry the PH20 retrotransposon. After transplantation of GI cells into an HI host, we can trace the fate of cells using this strain-specific probe (Kobayashi et al., 1999; Kato et al., 1999; Saito et al., 2003). Figure 4.2 shows a typical example of chimeric analysis during regeneration after transplantation. Using this system, we have succeeded in tracing the cells participating in regeneration. We observed that these cells migrate in the mesenchymal space to form the blastema and new tissues. We also demonstrated by combinatory experiments using an X-ray-irradiated host that the stem cells in the dorsal side can participate in the regeneration of ventral structures, suggesting that positional information resides in the differentiated cells, and that stem cells themselves may not possess persistent positional information (Kato et al., 2001). However, we have not yet succeeded in plantation and tracing of a single cell to demonstrate unequivocally the pluripotency of planarian stem cells.

4.3.3 Purification and Characterization of Stem Cells Using FACS

The most important progress in planarian stem cell studies was the development of a purification method for stem cells utilizing FACS (Hayashi et al., 2006). Exploiting the fact that planarian stem cells can be specifically eliminated by

X-ray irradiation, cell fractions specifically eliminated by X-ray irradiation were collected and characterized by using FACS. Figure 4.3 shows a comparison of cell sorting profiles between X-ray-irradiated and non-irradiated animals after dissociation into single cells and staining with a nuclear dye (Hoechst 33342) and a cytoplasmic dye (calcein). Two cell fractions were specifically eliminated by X-ray irradiation, and were named the X1 and X2 cell fractions. The cells in the X1 fraction showed high DNA and low cytoplasmic contents, suggesting that the X1 fraction may be composed of actively proliferating stem cells. In contrast to the X1 fraction, the cells composing the X2 fraction showed diploid DNA and low cytoplasmic contents, suggesting that this fraction may comprise non-proliferating X-ray-sensitive cells. To characterize each fraction, electron microscopic observation and gene expression analysis were conducted (Higuchi et al. 2007). The results clearly indicated that the two cell fractions may be composed of proliferating and resting stem cells, respectively. This was the first demonstration of the heterogeneity of planarian neoblasts. In addition, our studies revealed that the one-third of the cells comprising the X1 fraction showed slightly differentiated morphology, suggesting that cells may maintain the ability to proliferate after commitment. To test this possibility, we carefully checked the cells in intact animals using EM, and finally we thereby succeeded in identifying M phase-cells with differentiated cell features. We summarize these findings in Fig. 4.4. Our analyses clearly demonstrated the heterogeneity of “neoblasts”.

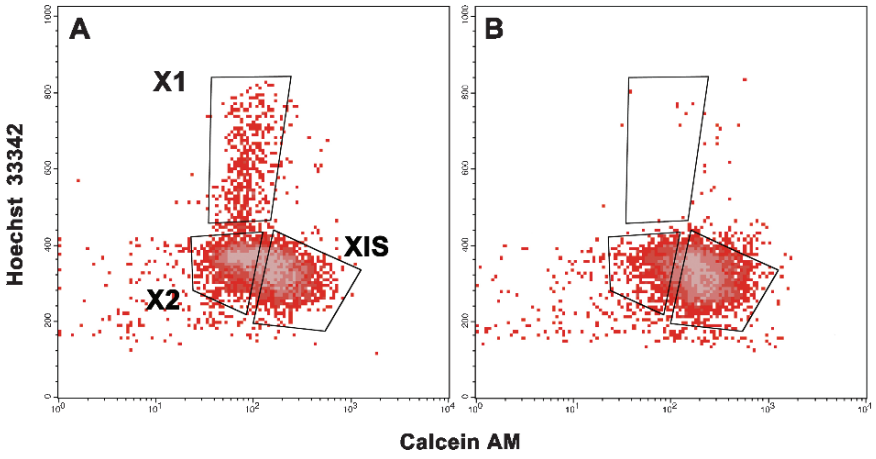

Fig. 4.3 Comparison of cell profiles between non-irradiated and X-ray-irradiated planarians after cell sorting using FACS. Two cell fractions named “X1” and “X2” were specifically eliminated by X-ray irradiation. (Hayashi et al., 2006)

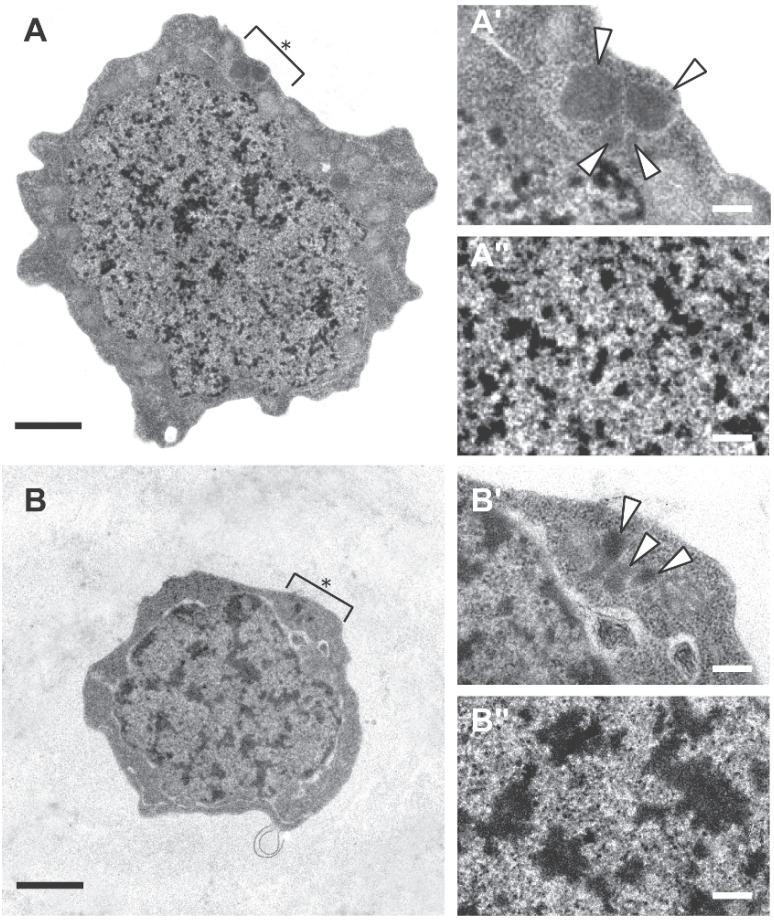

Fig. 4.4 Two types of planarian stem cells. (A) Micrograph of a stem cell enriched in the X1 fraction. (A') Higher magnification view of the chromatoid bodies (arrowheads) (A") Higher magnification view of the euchromatic nucleus. (B) Micrograph of a stem cell enriched in the X2 fraction. (B') Higher magnification view of the chromatoid bodies (arrowheads). (B") Higher magnification view of the heterochromatic nucleus. Scale bars: (A and B) 1 μm, (A', A", B' and B") 0.2 μm (Higuchi et al., 2007)

4.4 Recent Progress of Planarian Stem Cell Research: (II) Molecular Level

In the 1990s, molecular cloning using degenerate PCR primers became widely used in the field of planarian research. Homeobox-containing genes and other transcription factor genes were extensively isolated, but it was hard to detect their signals by in situ hybridization and to investigate their function by gene

manipulation (Orii et al., 1995, 1998, 1999; Umesono et al., 1997,1999; Koinuma et al., 2000, 2003). However, I succeeded in developing a detection method for RNA signals (Agata et al., 1998), and Sánchez Alvarado succeeded in developing a gene knockdown method using RNAi (Sánchez Alvarado and Newmark, 1999). These methodological improvements drastically modernized planarian research. Sánchez Alvarado and I also independently conducted EST projects, using *Schmidtea mediterranea* and *Dugesia japonica* (Mineta et al., 2003; Sánchez Alvarado et al., 2002). Sánchez Alvarado has now almost completed the genome sequencing of *S. mediterranea* (Robb et al., 2007). Use of these modernized techniques and DNA databases is leading to extensive accumulation of knowledge about the molecular basis of the planarian stem cell system.

4.4.1 Identification of Stem Cell-Related Genes in Planarian

The first molecular scalpel for dissecting the planarian stem cell system was produced by our group. We focused on the morphological resemblance of the chromatoid body and the germ plasm in other animals, and speculated that these structures may be composed of similar molecules. We also reasoned that if these structures contain the same components, this might provide a unique clue for investigating the molecular basis of pluripotent cells, since it would suggest that both the neoblasts and germ cells maintain pluripotency with germ granule-like materials. Based on these speculations, we succeeded in identifying the first neoblast-related gene, named *DjvlgA* (Shibata et al., 1999). *DjvlgA* was isolated as a homologue of the vasa gene of *Drosophila* using degenerate PCR primers. Vasa protein is known to be a major component of the germplasm of *Drosophila* oocytes. *DjvlgA* is expressed in X-ray-sensitive stem cells and their descendant cells. We found that *DjvlgA*-positive cells participated in blastema formation. Now, *DjvlgA* is used as a molecular marker for detecting blastema formation.

After these findings, many groups succeeded in identifying neoblast-related genes in a similar way. Genes for many homologues to *Drosophila* germplasm components, such as *piwi*, *bruno*, *pumilio*, *nanos*, and *Me31b*, were isolated and their expression in neoblast-like cells and germ cells was confirmed (Reddien et al., 2005; Rossi et al., 2006; Guo et al., 2006; Salvetti et al., 2005; Sato et al., 2006; Handberg-Thorsager and Saló, 2007; Wang et al., 2007; Yoshida-Kashikawa et al., 2007).

In particular, Sato et al. made very important findings using a *nano*s probe. *nanos* is specifically expressed in germline-committed stem cells, and unexpectedly, *nanos*- positive cells were detected in asexual-state as well as sexual-state planarians (Sato et al., 2006). Furthermore their analysis of the *nanos* mRNA distribution by EM in situ hybridization revealed that *nanos*-mRNA is specifically concentrated in the chromatoid bodies of prospective germline-committed stem cells. The *nanos*-positive cells showed completely identical morphology to the neoblasts, except for containing *nanos*-mRNA in their chromatoid bodies. These findings suggested that morphologically defined neoblasts may show heterogeneity

when they are analyzed at the molecular level. The chromatoid bodies may contain different mRNAs together with many common RNA binding proteins.

Although many homologues of germplasm components have been identified as stem cell-related genes, the relationship of the proteins encoded by these genes and the chromatoid body is still unclear. Only *nanos* mRNA and Me31b protein have been identified as components of the chromatoid body (Sato et al., 2006; Yoshida-Kashikawa et al., 2007). We speculate that the chromatoid body may be a huge complex of RNAs and RNA binding proteins that function to regulate the translation of mRNAs (Auladell et al., 1993; Agata and Watanabe, 1999). Studies of *nanos* have suggested that the chromatoid bodies themselves are heterogeneous, and their asymmetric distribution after cell division may have an important role in asymmetric cell fate determination of the stem cells (Sato et al., 2006). To make a breakthrough in this aspect of stem cell biology, we need to make more efforts to identify the molecular components of chromatoid bodies.

4.4.2 Identification of Genes Required for Stem Cell Maintenance

RNAi experiments of various stem cell-related genes have already been conducted. The most striking phenotype was obtained in *piwi*-homologue knockdown planarians. In *piwi*-homologue knockdown planarians, the stem cells in the X1 fraction were specifically eliminated, resulting in the loss of regenerative ability (Reddien et al., 2005). Recently, studies in *Drosophila* indicated that retrotransposons were activated in *piwi* mutants and killed the germline stem cells. PIWI may be a component of a complex required for inactivating retrotransposons, rather than a component of the chromatoid body. Gene knockdown planarians of *nanos* showed that *nanos* function is essential for the maintenance of germ cells but not for that of somatic stem cells (Handberg-Thorsager and Saló, 2007; Wang et al., 2007). A *bruno-like* gene *(bruli)* and *DjPum*, a homologue of *Drosophila pumilio* are required for the maintenance of both germline and somatic stem cells (Guo et al., 2006; Salvetti et al., 2006). However, we do not know whether Bruno and Pumilio are components of the chromatoid body or not. Not only genetic analysis, but also biochemical studies will be required to investigate the molecular basis of planarian stem cells.

4.5 Future Prospects

Recently, by developing new techniques, we have succeeded in rapidly accumulating new knowledge about planarian stem cells (Newmark and Sánchez Alvarado, 2002; Agata et al., 2003, 2006; Sánchez Alvarado, 2006; Rossi et al., 2007a, 2007b). Especially, recent studies have demonstrated the heterogeneity of planarian stem

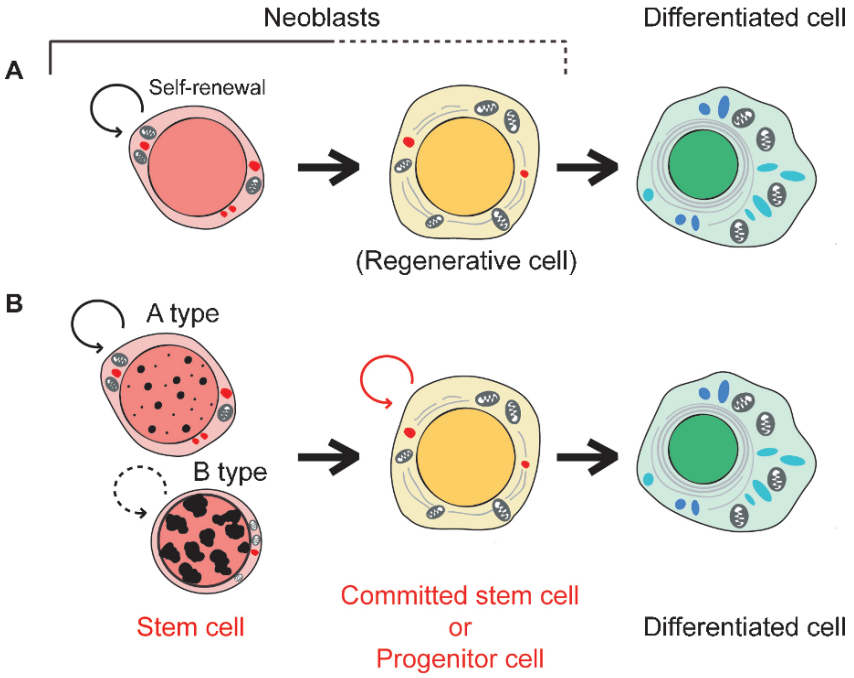

Fig. 4.5 The new view of the planarian stem cell system revealed by FACS analysis., (A) The classical view of the planarian stem cell system. (B)The new view of the planarian stem cell system obtained by FACS sorting and transmission electron microscopy analysis. The newly suggested aspects are indicated by the red words and arrow. Planarians may contain heterogeneous stem cells of at least three types (A, B, and committed/progenitor). Features of the cells are represented as: stem cell nucleus (red circle); heterochromatin (black region in nuclei), chromatoid bodies (small red circles), mitochondria (gray ovals); differentiating cell nucleus (committed/progenitor stem cell nucleus) (yellow circle); RER (gray lines); differentiated cell nucleus (green circle), other organelles (blue and aqua ovals). (Higuchi et al., 2007)

cells (Fig. 4.5), suggesting that planarians may have a stem cell system not unlike that of mammals. Thus, we expect to gain insight into many fundamentals of stem cell systems by studying planarian regeneration. However, we need to develop more advanced methods for investigating the pluripotency of planarian stem cells and the regulatory networks of the planarian stem cell system. Here I briefly discuss two critical approaches indispensable for planarian research.

4.5.1 Cell Culture

We will need to overcome several difficulties in order to develop a cell culture system for planarian stem cells. How can planarian stem cells be purified without damaging them? Can planarian stem cells be cultivated without any feeder cells? We developed an efficient planarian cell purification method using FACS. However,

for this method, the dissociated cells were stained with Hoechst nuclear dye to separate cells according to the difference of their DNA content, resulting in damage to the purified cells. Hoechst staining showed strong toxicity since we use highly concentrated Hoechst dye to stain dissociated cells under saturated conditions. When we stained cells using lower concentration conditions, we could not obtain a reproducible cell profiling pattern. After purification of the proliferating stem cell fraction (X1 fraction), we have never observed division of these cells under in vitro conditions; instead, they stayed in S-G2-M phase. When these cells were transplanted into X-ray-irradiated planarians, they did not survive, and induced a bubble structure inside of the hosts. Thus, we should develop another strategy for purifying stem cells without causing severe damage (unpublished observation).

We may have another problem for culturing planarian stem cells, since we have never observed dividing cells under in vitro conditions, even though dissociated cells were simply cultivated without using staining with any toxic dyes. The dissociated stem cells maintain their round shape without attaching to any substrate. These observations suggest that planarian stem cells may require feeder cells for survival and proliferation. In fact in the planarian body, neoblasts are always observed with mesenchymal cells. The identification of mesenchymal cells providing a niche for the stem cells may be essential for developing a planarian cell culture system.

4.5.2 Cell Fate Tracing by GFP Labeling

To investigate the behavior of individual stem cells in vivo, we should develop transgenic techniques, which would enable us to stably trace the cell fate at the single cell level, as has been done in hydra (Wittlieb et al., 2006). Many people have tried to develop gene transfer methods in planarians, using approaches such as electroporation, sonoporation, particle guns, and transfection using liposomes or virus vectors. Although foreign genes can be introduced inside of the cells using these methods, unfortunately, stable integration of these genes in the nuclear genome has never been observed. We also attempted gene transfer to the dissociated stem cells after purification by FACS and transplantation of the resultant cells into X-ray-irradiated planarians, but this approach did not work well. We must develop a method to make it possible to trace the cell fate of a single stem cell under in situ conditions in order to comprehend the power of planarian stem cells. We are looking forward to examining the behavior of GFP-labeled stem cells under in vivo conditions in the near future.

4.6 Concluding Remarks

Planarian research has rapidly advanced in the past decade thanks to the application of many modern techniques. However, the number of planarian researchers is still very low. We need more young researchers who have the power to overcome

technical difficulties. Planarian is a very unique animal for studying stem cell systems. I believe that young researchers who are fascinated by the high regenerative ability of planarians may achieve breakthroughs in stem cell biology.

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Chapter 5
The Stem Cell System of the Basal Flatworm *Macrostomum lignano*

Peter Ladurner[1], Bernhard Egger[1], Katrien De Mulder[1,2], Daniela Pfister[1], Georg Kuales[1], Willi Salvenmoser[1], and Lukas Schärer[3]

Abstract The scope of this review is to introduce the free-living flatworm *Macrostomum lignano* as an excellent model organism to address questions of platyhelminth stem cell biology. First, we sketch the historical origin of flatworm stem cell research. Second, we introduce *M. lignano*, and summarize the main advantages that we think it has over the classical planarian model. Third, we give a short summary of the simple culture techniques. Fourth, we give a detailed overview over its morphology and embryology as far as it is relevant for stem cell biology. Fifth, we summarize our main findings on stem cell biology, with respect to the identification of neoblasts, their distribution and number. We describe the ultrastructure of neoblasts, their dynamics and gene expression. Sixth, we outline ways to study sex allocation by means of stem cell labeling and manipulation. Last, we highlight the regeneration capacity of this species and link it to the stem cell system. We conclude that *M. lignano* is a highly suitable model organism to gain knowledge about flatworm stem cells and to provide insight into stem cell systems of higher organisms, including humans.

Keywords Platyhelminthes, neoblast, Planaria, evolution, regeneration

[1] Institute of Zoology, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria

[2] Department of Biology, University of Ghent, Ledeganckstraat 35, B-9000 Ghent, Belgium

[3] Evolutionary Biology, Zoological Institute, University of Basel, Vesalgasse 1, 4051 Basel, Switzerland

E-mail addresses

Peter Ladurner: Peter.Ladurner@uibk.ac.at, Bernhard Egger: Bernhard.Egger@uibk.ac.at, Katrien De Mulder: Katrien.DeMulder@ugent.be, Daniela Pfister: Daniela.Pfister@uibk.ac.at, Georg Kuales: Georg.Kuales@uibk.ac.at, Willi Salvenmoser: Willi.Salvenmoser@uibk.ac.at, Lukas Schärer: Lukas.Scharer@unibas.ch

T.C.G. Bosch (ed.), *Stem Cells: From Hydra to Man,*

5.1 Flatworm Stem Cells Rising

'Planarian regeneration displays such an array of astounding phenomena that they could very well force one to believe that planarian regeneration might be performed by witchcraft'

H.V. Brøndsted, 1969

Fascinated by the phenomenal regenerative capacity of flatworms Thomas Hunt Morgan performed a series of amputation experiments on planarian (tricladid) flatworms. Morgan found that tiny pieces of tissue could reorganize and regenerate to a complete adult worm. Morgan and contemporary workers like Harriet Randolph and Charles Manning Child laid the basis for flatworms as model organisms in regeneration research. More than half a century later tremendous progress had been made in elucidating the cellular, morphological and biochemical mechanisms of regeneration, as demonstrated in classical papers by Brøndsted, Chandebois, Dubois, Lender, Pedersen, Teshirogi, Wolff and many others (Brøndsted, 1969). Their contributions made clear that a pool of likely totipotent cells of embryonic character, generally called neoblasts, is responsible for regeneration. Building on this work many different research groups all over the world further contributed to establish flatworms in a larger scientific community, involving research on development, regeneration, and stem cell biology. The last decade has led to the establishment of several new flatworm research groups, and a shift towards more molecular approaches in the existing labs. This has led to the discovery of important signals and pathways regulating stem cells and regeneration.

Neoblasts – the flatworm stem cells – are truly remarkable. They are undifferentiated cells that can self-renew and it is assumed that they can differentiate into all cell types of the animal. They power the extraordinary capacity of flatworms to regenerate from tiny pieces of tissue and are responsible for cell renewal during homeostasis, development, growth, and regrowth after starvation. These fundamental properties are of great interest and raise a number of questions that are relevant not only for flatworm stem cells, but also for stem cell systems of higher organisms and humans: How can stem cells be identified? What is their number and distribution? What is the molecular regulation that keeps stem cells unspecialized? Do different subpopulations exist? How have stem cell systems and the germ line originated during evolution?

A better understanding of the nature of stem cell systems is calling for research involving different methodological approaches and a number of diverse organisms. For example, somatic tissues in adult *Drosophila* or *Caenorhabditis* are post-mitotic, therefore additional model organisms such as flatworms can contribute to the study of somatic stem cells. In the last decade the interest in neoblasts has increased, partly due to methodological advances. In this review we want to demonstrate the potential of *M. lignano* to serve as a potent system to study stem cell biology.

5.2 Why *Macrostomum lignano*

Macrostomum lignano (Fig. 5.1) (Ladurner et al., 2005b) is a marine, free-living flatworm, a basal member of the Rhabditophora, the largest taxon within the Platyhelminthes. Platyhelminthes are triploblastic, unsegmented and acoelomate worms lacking circulatory and respiratory organs (Rieger et al., 1991). They encompass a large range of free-living and parasitic flatworms and include important human parasites, such as the liver fluke *Fasciola hepatica*, the blood fluke *Schistosoma mansoni* – the causative organism of bilharzia, and the tapeworms (e.g. *Taenia*, *Echinococcus*).

Natural populations of *M. lignano* occur in the high-tide interstitial sand fauna on beaches of the Northern Adriatic Sea. *M. lignano* can be collected in the field from the sediment surface of sheltered beaches and exhibits a high ecological tolerance for both salinity and temperature in the field as well as in laboratory cultures.

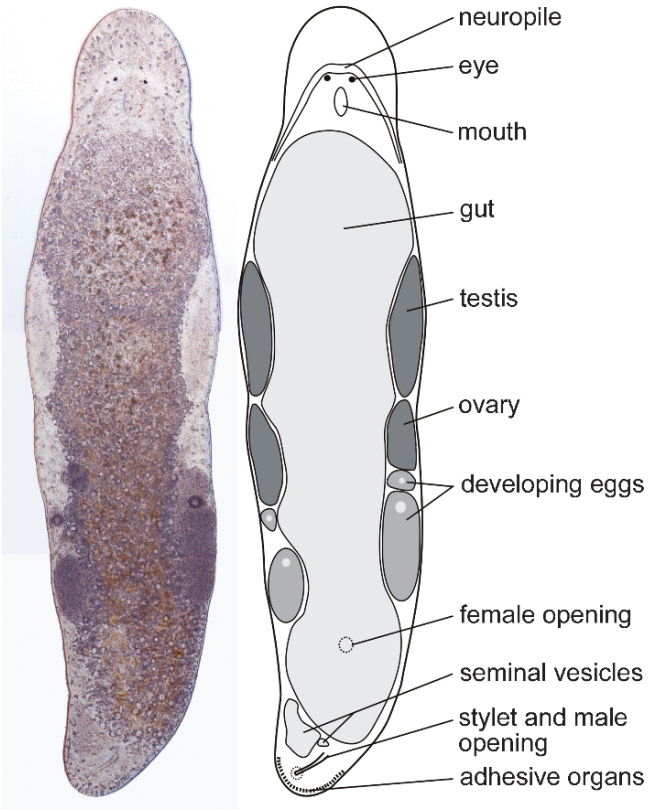

Fig. 5.1 Interference contrast photomicrograph of a living *Macrostomum lignano* and corresponding schematic drawing. The animal was relaxed and is slightly squeezed. The gut is filled with brownish diatoms, its food item. The length is about 1 mm

M. lignano is easily maintained in the laboratory. More than 100 different marine, brackish and fresh – water species of the genus *Macrostomum* occur worldwide. This diversity renders this taxon amenable to interdisciplinary approaches, particularly concerning questions related to evolution and development (evo-devo) and ecology and development (eco-devo). *M. lignano* is a non-self-fertilizing hermaphrodite that has been extensively used to study sex allocation, the question about how simultaneous hermaphrodites distribute energy resources to the male and female reproductive function (Schärer and Vizoso, 2007; Vizoso and Schärer, 2007; Schärer et al., 2004, 2007; Schärer and Ladurner, 2003) (see Section 5.6). The generation time is about 18 days. *M. lignano* possesses a substantial regenerative capacity (Egger et al., 2006, see Section 5.7).

We have established *M. lignano* as a complementary model organism for addressing fundamental questions of flatworm stem cell biology. Compared to planarians *M. lignano* has several important advantages: (1) *M. lignano* only consists of about 25,000 cells (Ladurner et al., 2000), which is about two orders of magnitude less than an adult planarian worm. Moreover, the same cell types, tissues and organs are present in both groups, but their complexity and organization is much simpler in *M. lignano* (Ladurner et al., 2005b). In addition, a number of monoclonal antibodies are available for staining specific cell types and tissues (Ladurner et al., 2005a). (2) *M. lignano* is very small, about 1.5 mm long. This greatly simplifies the observation of whole mount preparations using high resolution objectives. (3) Although *M. lignano* appears brownish under a dissecting microscope, it is highly transparent when viewed under a compound microscope, particularly when using interference contrast imaging. In squeeze preparations all major tissues and organ systems can be easily observed and quantified (Schärer and Ladurner, 2003). During such observations the animals are not harmed, allowing detailed and repeated observations, for example of regenerating tissues, over extended periods of time (Egger et al., 2006). Under high magnification it is possible to observe intricate morphological details of various gland cell types, spermatogonia, sperm, oogonia, developing and mature eggs, the copulatory stylet, adhesive organs, and even single stem cells in living worms. For studies of sexual selection even the amount of received sperm in the female sperm storage organ can be quantified (Schärer and Ladurner, 2003). (4) In contrast to planarians, which exhibit a highly derived embryogenesis (Cardona et al., 2006; Cardona et al., 2005) sometimes referred to as 'blastomereanarchy', the early embryonic cleavages of *M. lignano* are of the quartet spiral type (Morris et al., 2004). (5) *M. lignano* possesses compact testes and ovaries that are delineated by thin somatic tunica cells. This greatly facilitates the interpretation of whole-mount gene expression studies. In sexual planarians, on the other hand, the reproductive organs are dispersed throughout the body of the worm with numerous follicular testes, small ovaries and yolk producing vitellaria (Rieger et al., 1991). (6) Morphological and molecular analyses reveal that *M. lignano* is one of the basal-most lophotrochozoan species (Philippe et al., 2007). Its availability for laboratory studies thus permits to address questions related to the origin and evolution of stem cell systems.

In summary, the morphological, developmental and evolutionary characteristics of *M. lignano* allow a multitude of powerful approaches to elucidate cellular and molecular mechanisms of flatworm stem cell biology.

5.3 Culturing *Macrostomum lignano*

M. lignano is very easy to maintain in the laboratory. For long-term cultures animals are generally kept in glass petri dishes in f/2, a nutrient-enriched artificial seawater medium at a salinity of 32‰ (Andersen et al., 2007; Egger and Ishida, 2005). These dishes are kept in a climate chamber at a constant temperature (20°C), with a 14 h:10 h or 13:11 h light:dark cycle and at 60% relative humidity (Schärer et al., 2007a; Egger and Ishida, 2005). In the laboratory, *M. lignano* can survive in artificial seawater from salinities as low as 1‰ to as high as 40‰ Temperatures may vary between refrigerator conditions (4°C) to 40°C or more, reflecting the capacity to survive cold winter environments and the heat of the sand surface in summer.

Animals are fed with the diatom *Nitzschia curvilineata* that can be obtained from an algae stock center (strain 48.91, http://sagdb.uni-goettingen.de/) and that is propagated and grown under the same conditions as the worms. For algae growth strong fluorescent lights of an appropriate light colour are required (e.g. Osram 36W/ 12-950). In the field the worms feed on a variety of different algae, but in the laboratory they can be kept on this single species, thus greatly simplifying the culturing. As they lack an anus the worms expel rod-shaped ‘faeces’ containing siliceous cell walls of partly-digested diatoms through the mouth.

5.4 Morphology and Embryology of *Macrostomum lignano*

5.4.1 Morphology

Macrostomum lignano is covered by an epidermis of multiciliated cells that are primarily responsible for locomotion. Under the epidermal layer and a thin extracellular matrix a complex muscle system consisting of circular, diagonal, and longitudinal muscle cells is present (Ladurner et al., 2005a; Salvenmoser et al., 2001). The bilaterally symmetrical nervous system consists of a brain with a neuropile surrounded by perikarya, two main lateral nerve cords connected by a postpharyngeal commissure and a caudal loop, and two additional dorsal and ventral nerve cords (Egger et al., 2007; Bode et al., 2006; Nimeth et al., 2004). Peripherally, a nerve-net like plexus covers the animals and contains sensory nerve cells. Light perception by photosensitive cells occurs within the two pigment-cup eyes. Adults as well as juveniles, swim away from the light (negative phototaxis). The acoelomate body is

filled with parenchyma – the tissue between the body wall and the gut. It is narrow and compact and contains various gland cell types. Moreover, it also contains the stem cells and cells in the process of differentiation. *M. lignano* possesses a pharynx simplex coronatus leading to the sac-like gut, which is completely lined with ciliated gastrodermal cells. Excretion and osmoregulation is achieved by flame bulb cells attached to the protonephridial canals. The chromosome number of *M. lignano* is 8 (2n), two more than in most other macrostomid species studied so far. This may indicate that in *M. lignano* one chromosome pair has either undergone duplication or fission (Egger and Ishida, 2005). The reproductive system consists of paired testes located laterally in the central region of the animal and posterior to them there are paired ovaries and developing eggs. Sperm are accumulated in the seminal vesicles and transferred with the stylet to the copulation partner (Schärer and Vizoso, 2007; Schärer et al., 2007; Ladurner et al., 2005b).

5.4.2 Embryology

A detailed analysis of *M. lignano* embryogenesis was given by Morris et al. (2004). Briefly, embryonic development of *M. lignano* takes about 5 days. The first three rounds of divisions follow the typical spiral cleavage pattern. Later vegetal cells stretch out to form hull cells (called Hüllzellen by Seilern-Aspang 1957), which cover the other blastomeres. Embryonic primordia are formed by proliferation of internal cells. These cell clusters expand and give rise to primordia of the body wall, the nervous system and the gut. After about 2.5 days the onset of tissue and organ differentiation begins and the definitive epidermis is formed. Later, pharynx and a ciliated gut are developed and a neuropile is formed in the center of the bilateral brain. In the next developmental stage the orthogonal muscle grid becomes completed and the embryo elongates within the eggshell. The brain primordium condenses further and eyes are formed. Finally, a small worm of about 3,000 cells and 250 µm in length hatches from the eggshell and immediately starts to ingest food. Moreover, the number of circular and longitudinal muscles increases three- to five-fold postembryonically, additional nerve cells differentiate (Ladurner et al., 2005a), the gut elongates and the total cell number of the animal rises to about 25,000 (Ladurner et al., 2000) for the adult. Up to now, the embryonic origin of stem cells remains unknown.

5.5 The *Macrostomum lignano* Stem Cell System

'The fundamental problem as to the origin of the neoblast and its relation to the parenchyma cells is very difficult to solve'

Pedersen, 1959

5.5.1 Identification of Neoblasts

M. lignano neoblasts have the typical characteristics of flatworm neoblasts. Neoblasts are small (6–10 µm in diameter), have a high nuclear/cytoplasmic ratio, a large nucleus with a prominent nucleolus, a thin rim of cytoplasm with free ribosomes, few mitochondria, and none or very little endoplasmic reticulum (Bode et al., 2006; Nimeth et al., 2002; Peter et al., 2001; Ladurner et al., 2000; Rieger et al., 1991, 1999). In planarians and parasitic flatworm species neoblasts of similar morphology have been described (Newmark and Sànchez Alvarado, 2000; Hori, 1997; Morita, 1995; Gustafsson and Eriksson, 1992; Palmberg, 1990; Baguñà, 1981; Gustafsson, 1976; Pedersen, 1959; Wolff and Dubois, 1948).

In *M. lignano* neoblasts can be observed using light microscopy in squeeze preparations of live animals (Fig. 5.2D, Rieger et al., 1999). By applying maceration techniques single cell suspensions can be obtained and numerous different cell types including neoblasts can be observed (Peter et al., 2001; Ladurner et al., 2000). In recent years 5'-bromo-2'-deoxyuridine (BrdU) labeling has been adapted to label and trace the S-phase subpopulation of neoblasts in *M. lignano* using whole mount immunocytochemistry, histological sections, and immunogold staining (Nimeth et al., 2002, 2004, 2007; Egger et al., 2006; Bode et al., 2006; Peter et al., 2001; Ladurner et al., 2000). Moreover, the mitotic subset of neoblasts can be visualized by applying a polyclonal antibody against phosphorylated Histone H3 (Nimeth et al., 2004, 2007; Bode et al., 2006; Ladurner et al., 2000). A number of conserved genes known to play a role in stem cell maintenance or proliferation have been isolated from *M. lignano* (Pfister et al., 2007) and can be used to identify neoblasts (see below). Moreover, a protocol for a large-scale whole mount *in situ* hybridization screen has been established (Pfister et al., 2007). This allows to systematically screen for new candidate marker genes based on the annotated ESTs of *M. lignano* (Morris et al., 2006).

5.5.2 Distribution and Number of Neoblasts

For macrostomids, the earliest evidence for a bilateral distribution of neoblasts was provided by Rieger et al. (1994) in an ultrastructural reconstruction of serial sections of the caudal region of *Macrostomum hystricinum marinum* (Fig. 5.2A). It became apparent that neoblasts were clustered on the lateral sides of the animals in close relation to the main lateral nerve cords. This was later confirmed for S-phase (Fig. 5.2B) and mitotic neoblasts (Fig. 5.2C) identified by immunohistochemistry (Nimeth et al., 2004, 2007; Egger et al., 2006; Bode et al., 2006; Peter et al., 2001; Ladurner et al., 2000), ultrastructure (Bode et al., 2006; Rieger et al., 1999), by interference contrast (Fig. 5.2D), by histological staining (Fig. 5.2E), and molecular markers (Pfister et al., 2007). In summary, in *M. lignano* two bands of neoblasts are located along the lateral sides of the animal and merge in the tail plate. Somatic neoblasts are

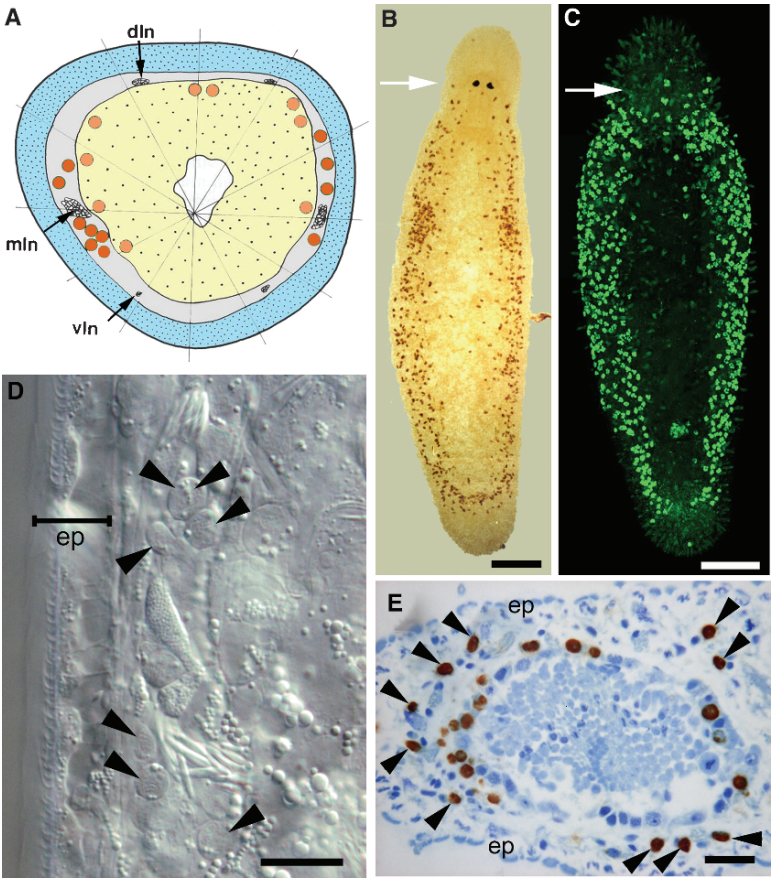

Fig. 5.2 Distribution of neoblasts. (A) Reconstruction of ultrastructural serial sections (6 µm) of *Macrostomum hystricinum marinum*. Epidermis (blue), gastrodermis (yellow), mesodermal parynchyme (gray) in between. Note localization of mesodermal neoblasts (orange) at the lateral sides close to the main lateral nerve cords (mln). dln, dorsal nerve cords; vln, ventral nerve cords. Gastrodermal neoblasts (light orange) can be found also along the midline. (Modified after Rieger et al., 1994, Zoomorphology, Vol. 114, p. 140, Fig. 5.9C, with kind permission of Springer Science and Business Media.) (B) S-phase cells (brown spots) after 30 min BrdU visualized with horseradish peroxidase. Arrow indicates level of the eyes. (Reprinted from Bode et al., 2006, Cell Tissue Res., Vol. 325, p. 580, Fig. 5.2A, with kind permission of Springer Science and Business Media.) (C) Mitoses (anti-phos. H3) accumulated after 24 h using Colchicine to arrest mitoses in metaphase. Arrow indicates level of the eyes. (Reprinted from Ladurner et al., 2000, Dev. Biol. Vol. 226, p. 235, with permission from Elsevier.) (D) Interference contrast image of a squeeze preparation of a live animal. Note neoblasts (arrowheads) below the epidermis (ep). (E) Lateral sagittal section through the testis of an animal treated for 30 min with BrdU. Note mesodermal neoblasts (arrowheads) and spermatogonia and spermatocytes stained with horseradish peroxidase. The testis center (compact mass of cells) containing differentiating spermatides and spermatozoa does not contain proliferating cells. ep, epidermis. Scale bars (B, C) 100 µm, (D) 10 µm, (E) 20 µm

also present along the postpharyngeal commissure. A few, most likely gastrodermal stem cells, can be found scattered along the midline of the body. In the epidermis and in the rostrum, the region anterior to the eyes, neoblasts are entirely lacking.

The determination of the number and distribution of neoblasts in flatworms has interested researchers since the discovery of neoblasts. In his review on planarians Brønsted (1969) stated that '*many clues as localization of neoblasts occur in the literature, especially their topographical connection with the lateral nerve trunks, but few workers have had the patience to count them*' (p. 114). By and large an overall pattern became apparent that neoblasts were present in the parenchyma of the trunk and the tail of different triclad species but were generally lacking in the region anterior to the photoreceptors and in the pharynx. This view was corroborated by analyses using macerated preparations of different body parts (Baguñá and Romero, 1981; Baguñà, 1976a, 1976b), BrdU labeling and staining of mitoses (Ladurner et al., 2000; Newmark and Sánchez, Alvarado, 2000) and molecular markers (Rossi et al., 2006, 2007; Cebria et al., 2002, 2007; Higuchi et al., 2007; Kobayashi et al., 2007; Salo, 2006; Reddien et al., 2005; Salvetti et al., 2000, 2005; Newmark, 2005; Agata, 2003; Sánchez Alvarado et al., 2002).

For *M. lignano*, we have determined neoblast numbers and subsets of neoblasts using BrdU for S-phase neoblasts (Fig. 5.3A–E) (Ladurner et al., 2000), anti-phosphorylated Histone H3 (mitosis and meiosis) and ultrastructural serial sectioning including immunogold staining to estimate the total neoblast number. About 1,600 cells were assigned as neoblasts by ultrastructural characteristics (Bode et al., 2006) and approximately one-quarter of all neoblasts (about 400–500) are in S-phase at any moment and only a small fraction of about 20–40 cells is in mitosis.

The percentage of neoblasts compared to the total cell number was described for different flatworms species and appears to vary greatly, ranging from 6.5% in *M. lignano* (Bode et al., 2006) to 44% in *Dugesia tahitiensis* (Peter et al., 2001). These figures have to be considered in relation to the methods that were applied to label and identify neoblast-like cell types. Ultrastructure is probably best suited for recognizing neoblasts of an exclusively undifferentiated nature, while other methods may include neoblast stages of gradually increasing states of differentiation.

5.5.3 Ultrastructure of M. lignano Neoblasts

The fine structure of *M. lignano* neoblasts resembles stem cells of other flatworms. In a light and electron microscopical survey on the morphology of *M. lignano* neoblasts Bode et al. (2006) identified mesodermal, gastrodermal, and gonadal types of stem cells. Based on their cytoplasmic and nuclear structure each respective type could be further subdivided into different stages of gradually increasing differentiation. In adult *M. lignano* mesodermal neoblasts of stage 2, an intermediate stage 2–3 and of stage 3 were distinguished. Immunogold labeling after 30 min BrdU labeling revealed that in adult *M. lignano* stage 1 neoblasts were not detected, one third of all observed neoblasts were in stage 2, about 46% in stage 2–3 and 21%

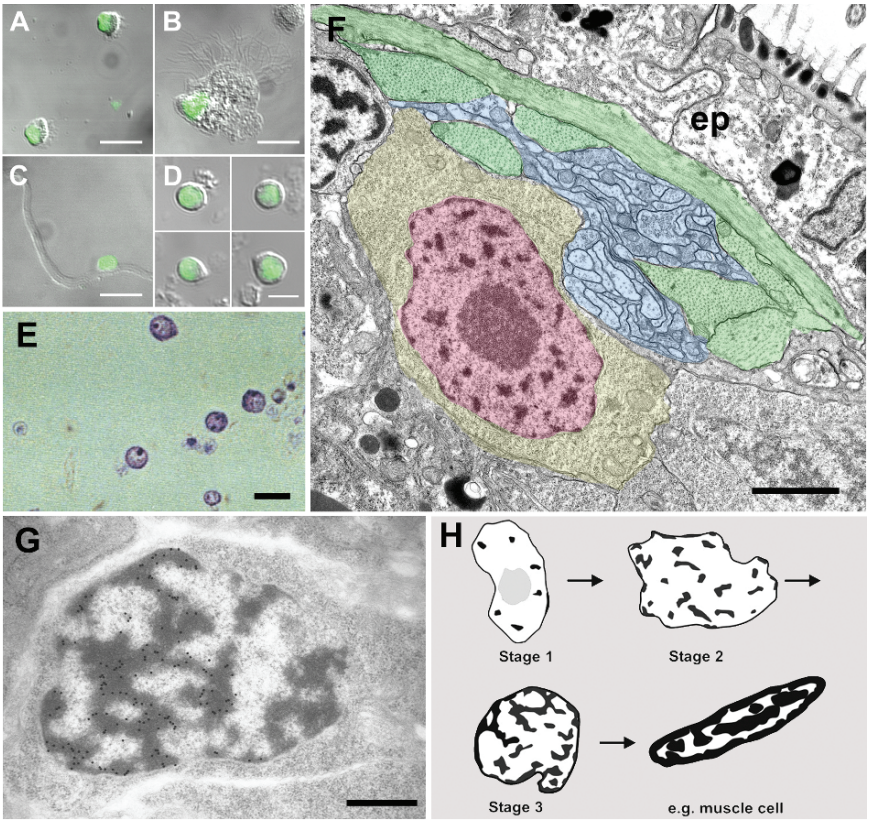

Fig. 5.3 Morphology and identification of *M. lignano* neoblasts. (A–D) Cells of macerated BrdU labeled animals; BrdU labeled nuclei are stained green. Reprinted from Ladurner et al., 2000, Dev. Biol. Vol. 226, p. 237, with permission from Elsevier (A) Epidermal cells, (B) gut cell, (C) muscle cell after continuous 14-day exposure to BrdU. (D) Neoblasts after a 30-min BrdU pulse (E) Neoblasts in cell suspension of disintegrated *M. lignano*. (Reprinted from Bode et al., 1999, Invertebr. Reprod. Dev., Vol. 35, p. 131, Fig. 5.3, with kind permission of Springer Science and Business Media.) (F) Ultrastructure of a neoblast with nucleus (red) containing patchy clumps of condensed chromatin, surrounded by a thin rim of cytoplasm (yellow). The neoblast is in direct contact with the main lateral nerve cord (blue) located below the body wall musculature (green) and the epidermis (ep). (G) Immunogold of a BrdU labeled neoblast. Note that gold particles are located on condensed chromatin. (H) Schematic drawing of neoblast nuclei with increasing complexity of chromatin structure. Scale bars (A–E) 10 µm, (F, G) 2 µm

in stage 3. The increase in cytoplasmic specialization led to the suggestion that stages 1–3 reflect sequential stages of differentiation. Stage 1 neoblasts were only found in young hatchlings and in the regeneration blastema. They are described as small spherical or oval cells with a thin rim of cytoplasm containing free ribosomes and mitochondria but lacking any signs of cytoplasmic differentiation. The nucleus contains individual small spots of condensed chromatin and usually a large nucleolus is present. Along the nuclear envelope small patches of condensed chromatin

are located. Stage 2 (Fig. 5.3F) neoblasts are characterized by an oval or elongated nucleus containing small independent clumps or short strands of condensed chromatin. Along the nuclear lamina a thin stratum or small accumulations of condensed chromatin can be found. In the cytoplasm free ribosomes and small strands of rER are present. Stage 3 neoblasts (Fig. 5.3G) possess thicker bands of condensed chromatin and only few independent clumps. The nuclear envelope is well established and in the cytoplasm rER and dictyosomes are present. Intermediate morphologies are referred to as stage 2–3. Mesodermal neoblasts possess small chromatoid bodies in the cytoplasm. Gastrodermal neoblasts are found to be situated at the base of the gut epithelium. Their overall morphology is comparable to mesodermal neoblasts but larger clumps of condensed chromatin compared to mesodermal neoblasts are present in stage 3 gastrodermal stem cells. Spermatogonia and oogonia exhibit a chromatin morphology akin to stage 1 or stage 2 neoblasts but possess multiple small chromatoid bodies and a cytoplasm without any signs of differentiation. A schematic drawing of stage 1 to stage 3 neoblast chromatin organization is shown in Fig. 5.3H.

5.5.4 Neoblast Dynamics

5.5.4.1 Cell Cycle

BrdU labeling in combination with mitotic staining and the experimental arrest of neoblasts using Colchicine has enabled the identification of neoblast subpopulations with different cell cycle rates. More than 13% of the cycling neoblasts propagate from S-phase into mitosis within 2 h, 52% within 4 h and about 89% within 24 h, respectively. Mitotic cells accumulate with a rate of 8 cells/h over a 24 h period when Colchicine is applied. In untreated animals a number of 26.8 ± 8.4 mitotic cells are present when animals are processed for immunostaining using an antibody against phosphorylated Histone H3 (Nimeth et al., 2004).

During starvation animals decrease in size and regress the reproductive organs. The number of mitotic cells declines. After 30 days of starvation mitotic figures are almost lacking. However, feeding such animals induces a rapid and dramatic increase in the number of mitoses. Probably neoblasts arrested in G2 enter mitosis and contribute to a fast boost of mitoses. After the depletion of the G2 pool of neoblasts the number of mitoses decreases followed by another increase of mitotic figures backed by cells that have passed through S-phase. Starvation of 3 months results in animals similar to hatchlings, lacking any reproductive organs (Nimeth et al., 2004). After feeding animals completely recover adult morphology.

Blocking neoblasts entering S-phase using hydroxyurea leads to a strong reduction in the number of S-phase cells and a decrease in the intensity of staining. After 7 days of hydroxyurea treatment some gonadal stem cells but not somatic stem cells do incorporate BrdU (Nimeth et al., 2004). Animals recover the normal proliferation pattern if transferred to culture medium lacking hydroxyurea, suggesting that

the effect of hydroxyurea is transient. Pfister et al. (2007) observed that after 11 days of hydroxyurea treatment the expression of *vasa* remained in ovaries but was lacking in testes.

5.5.4.2 Cell Renewal

The renewal of differentiated cells was observed by continuous BrdU labeling of animals up to 14 days. In a first estimation Ladurner et al. (2000) described the renewal of about one third of all epidermal cells within 14 days. By analyzing confocal projections of such worms Nimeth et al. (2002) estimated that about 7,000 cells (of the total of 25,000) were replaced. This requires a daily renewal of about 500 cells, i.e. about 21 cells per hour. Apoptosis was studied in *M. lignano* using TUNEL, annexin labeling and ultrastructure in order to address the question on how the balance between cell proliferation and cell loss was achieved (Nimeth et al., 2002). However, it was found that the observed number of apoptotic figures was insufficient to explain tissue homeostasis in *M. lignano*. Apparently apoptosis-independent mechanisms were involved or epidermal cells were sloughed off directly from the epidermal sheath.

5.5.4.3 Neoblast Migration

Two main routes for migrating neoblasts have been described for *M. lignano* (Ladurner et al., 2000; Nimeth et al., 2004). In BrdU pulse-chase experiments a 30 min BrdU pulse was applied and the fate of the labeled neoblasts was followed after different chase times. In such experiments neoblasts can be observed to migrate from the region lateral to the eyes into the rostrum – the region anterior to the eyes (that lacks any proliferating cells). It was estimated that neoblasts migrate with a rate of 6.5 µm/h. Nimeth et al. (2004) quantified these migrating neoblasts and showed that in Colchicine treated animals 7.0 ± 3.0 neoblasts migrate into the rostrum after 16 h and 15,6 ± 6,9 neoblasts after 24 h, respectively. Ladurner et al. (2000) observed that neoblasts lateral to the eyes divide frequently and pairs of BrdU labeled cells, oriented in a anterior-posterior fashion, were present after 4 h of chase time, indicating that the (anterior) daughter cell migrated away form the (posterior) neoblast towards the rostrum. A 30 min BrdU pulse never showed such cell pairs, which strongly suggests that these pairs originate from recent cell divisions. Division and migration of these neoblasts were also analyzed by immunogold labeling and this revealed that the posterior neoblast always showed a stage 2 phenotype while the anterior cell exhibited a stage 3 morphology.

A second route of neoblast migration occurs from the lateral sides where most S-phase and mitotic neoblasts are located (Ladurner et al., 2000) towards the dorsal and ventral median plane. Ultimately, neoblast migration and differentiation leads to a completely homogenous pattern of epidermal cell renewal (see Ladurner et al., 2000; Nimeth et al., 2002; Bode et al., 2006).

5.5.4.4 Elimination of Neoblasts by Irradiation

Attempts have been made to eliminate the stem cell pool by gamma-irradiation. Doses up to 80 Gray have not been sufficient to abolish the stem cell pool and expression of the stem cell and germ line marker *macpiwi* was still present after 3 weeks post irradiation (Pfister et al., 2007). In following experiments intensities of a single radiation pulse up to 200 Gray (Ladurner, unpublished data) or repeated irradiations with lower doses adding up to 150 Gray over 2 or 8 days did not result in a complete elimination of proliferating cells or gene expression (De Mulder, unpublished data). About 10% of all animals recovered cell proliferation as well as the expression of stem cell- or housekeeping genes after 21 days post irradiation. Apparently quiescent neoblasts and/or a potent DNA repair system contributed to the survival of these animals. Further experiments will be necessary to obtain stem cell free hosts. Such animals can be used for e.g. transplanting purified neoblasts from non-irradiated donors to analyze the differentiation potential of *M. lignano* neoblasts as was shown for planarians (Baguñà et al., 1989).

5.5.5 Molecular Approaches to M. lignano Stem Cells

A number of different plasmid and phage libraries were produced for *M. lignano* (Morris et al., 2006). From a plasmid library we have sequenced 7,680 *M. lignano* EST clones of which 68% are similar to previously reported genes of known function (Morris et al., 2006). An annotated database of the *M. lignano* EST collection has been made available on the web (http://macest.biology.ucla.edu/macest/). The database can be browsed according to annotated categories or BLAST searches can be performed against the database. In addition, 5,000 ESTs have been sequenced from a *M. lignano* phage library. These sequences have just become available and have not yet been analyzed or annotated in detail. Molecular tools such as *in situ* hybridization, RNA interference (Pfister et al., submitted; Pfister et al., 2007), or microsatellites (Schärer, unpublished data) have been developed. A number of candidate genes involved in stem cell regulation, such as *piwi-, vasa-, PCNA-, MCM2-,* or *pumilio*-like genes, have been isolated using degenerate primers or were identified in a high throughput screening (Pfister et al., 2007). To facilitate protein localization polyclonal antibodies for most of these genes have been developed (Fig. 5.4A–D).

For example, a *vasa*-like orthologue is expressed in a subset of somatic neoblasts and in the gonads (Fig. 5.4E) and its expression and protein localization was followed during postembryonic development, regeneration and starvation (Pfister et al., submitted). In *M. lignano*, a *piwi*-like gene is expressed in somatic stem cells and gonadal stem cells (Fig. 5.4F) and was shown to be eliminated from somatic stem cells and testes but not from the ovaries after gamma irradiation. Functional knock-down of *M. lignano piwi* results in the loss of somatic neoblasts, the degradation of *piwi* mRNA and protein and the death of the animals (De Mulder, unpublished

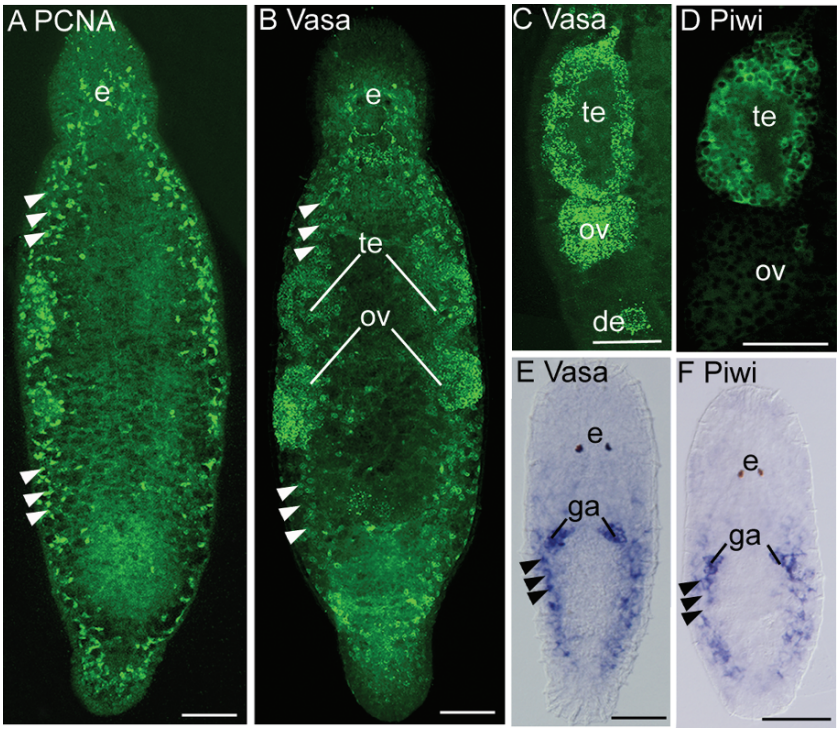

Fig. 5.4 Immunocytochemistry (A–D) and *in situ* hybridization (E, F) using stem cell markers. Antibodies were raised against *M. lignano* PCNA (A), Vasa (B, C), and Piwi (D). (A, B) Confocal projection of the dorsal side. Proliferating neoblasts are located at the lateral sides. Arrowheads indicate stained neoblasts. e, eyes; te, testes; ov, ovaries. (C, D) Confocal projection of the gonads. te, testes; ov, ovaries; de, developing eggs. Note that both, Vasa and Piwi are present only in the outer margins of the testes that contain spermatogonia and spermatocytes. Mature sperm are in the centre of the testes that is not stained. (E, F) *In situ* hybridization using *vasa* (E) and *piwi* (F) probes of *M. lignano* hatchlings. Note staining of the gonad anlage (ga) and neoblasts (arrowheads). e. eyes. All scale bars 75 µm

data). *PCNA* seems to be present in proliferating cells and can be applied as marker if the denaturation step of the BrdU staining protocol needs to be avoided. Additional potential stem cell markers like e.g. *annexin11, musashi, Histone H2A.Z* are currently being evaluated.

5.6 Stem Cells and the Evolutionary Biology of Reproduction

The accessibility of the stem cell system of *Macrostomum lignano* also allows interesting approaches to study the economy of reproductive allocation, a topic of great practical and theoretical interest in evolutionary biology. All sexually

reproducing organisms need to decide on a) the allocation towards reproduction versus maintenance in the context of their life histories (Stearns, 1992) and b) the allocation of resources towards male versus female reproduction, a process called sex allocation (Charnov, 1982). Higher allocation to a particular reproductive function will in many cases be linked to a higher stem cell proliferation rate in the particular organs. Worms that are grown in large group sizes were shown to react by increasing the size of their testes (Schärer and Ladurner, 2003), presumably to counteract the higher sperm competition in larger groups. Using the BrdU labeling technique it was subsequently shown that this increase in testis size is linked to a higher number of proliferating stem cells in the testes (Schärer et al., 2004), thereby showing that bigger testes do work more.

Current efforts aim at using and extending the knowledge about testis- and ovary-specific gene expression patterns to gain experimental control over the allocation of resources to these organs. RNAi knockdown experiments of such genes may allow to produce individuals with an experimentally manipulated sex allocation, or in extreme cases even males or females in this normally simultaneously hermaphroditic flatworm. This will allow interesting experiments regarding mating role preferences and sexual conflict (Michiels, 1998; Charnov, 1979).

Moreover, an interesting field for future research will be the role of stem cells versus somatic cells in the establishment of certain resource allocation patterns. Based on the analyses of different isofemale lines there is evidence that both testis and ovary size in *M. lignano* are at least in part genetically determined (L. Schärer and D. Vizoso, unpublished data). It is thus interesting to ask if these allocation patterns are caused by the genetics of the somatic environment, by the stem cells, or by an interaction of the two. This question could be addressed by transplanting germ line stem cells of one isofemale line into the somatic background of another and determining the resulting allocation patterns.

5.7 Regeneration in *Macrostomum lignano*

Macrostomum lignano is able to regenerate missing body parts anteriorly, posteriorly and laterally, but pieces without brain and the major part of the pharynx fail to regenerate the whole body. Adult worms are able to regenerate a second head including brain and pharynx, but only in the presence of the old head, which indicates that the nervous system plays a major role during regeneration in *M. lignano*, notably the brain and the large nerve clusters besides the pharynx. The limited regeneration capacity in *M. lignano* and many other flatworms may therefore be attributed to the lack of stimuli from the nervous system, and not to an inherent limited potency of neoblasts (Egger et al., 2007; Egger et al., 2006).

Both adults and juveniles can regenerate. Complete regeneration is possible from pieces as small as 4,000 cells for adults and 1,500 cells for freshly hatched juveniles. In these fragments only about 160 and 50 neoblasts are present, respectively. Such a small number of neoblasts is enough to give rise to all cell types required

for regeneration of a complete animal – the lowest number known for any bilaterian animal (Egger et al., 2006).

Within 2–3 weeks, adult animals amputated behind the pharynx are able to quintuple their cell number to reach that of a fully grown adult. Repeated regeneration of the same individuals was observed for up to 59 amputations during a period of more than 2 years (Egger et al., 2006). These experiments suggest that not only differentiated cells, but also stem cells themselves are being replenished upon regeneration.

At certain cutting levels the gonads are completely removed by amputation, but will be regenerated. These regenerates are able to produce fertile offspring. Thus somatic neoblasts have the ability to reconstitute the germline. Similarly, somatic neoblasts from the mesodermal space can reconstitute the whole gut including gastrodermal stem cells.

A distinct blastema, i.e. an accumulation of undifferentiated neoblasts and differentiating cells, can be distinguished as early as 24 h after amputation of the posterior part of the animal. At this time, the overall number of neoblasts in S-phase has significantly exceeded that of control animals, and especially the blastema is comprised to a large extent of S-phase neoblasts (Fig. 5.5B–C). While the overall number of mitoses decreases during the first 8 h, it reaches a peak at

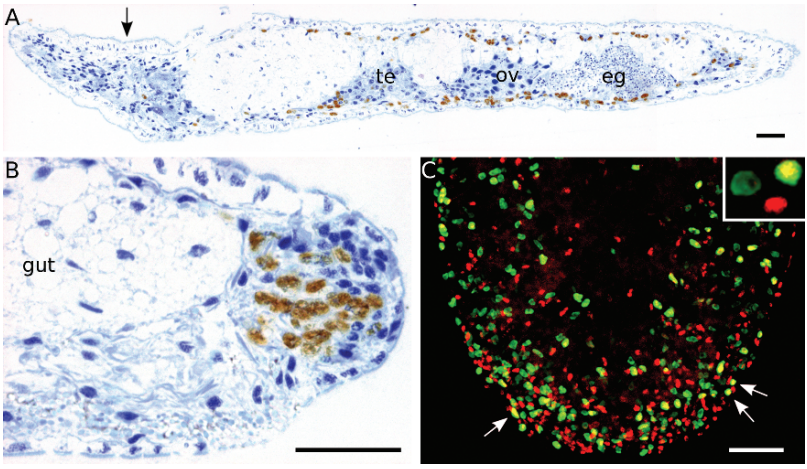

Fig. 5.5 Regeneration of *M. lignano*. (A) Sagittal serial section of an animal 24 h after amputation behind the female opening. S-phase neoblasts (brown) are mainly distributed laterally along the ventral main nerve cords and the dorsal nerve cords. The blastema is only visible in later sections of the series. Arrow indicates the level of the eyes. te testis, ov ovary, eg developing eggs (B) Detailed view of a later section of the same series as (A). The blastema in the posterior is comprised of neoblasts (brown ones in S-phase) and cells in early differentiation. (C) Double labeling of a 24 h regenerant with two different thymidine analogues. A iododeoxyuridine pulse (red) was chased for 24 h, after which a chlorodeoxyuridine pulse (green) was applied. Double-labeled nuclei are yellow, some of which are denoted with arrows. Inset shows single- and double-labeled nuclei. Horizontal confocal projection. Anterior in panels (A) and (B) is left, in panel (C) at the top. All scale bars 25 µm

48 h, when the blastema has formed a large bulge. Interestingly, the time needed for fast-cycling neoblasts to pass from S-phase to mitoses cannot be decreased during emergencies such as regeneration (Nimeth et al., 2007; Egger et al., 2006; Salvenmoser et al., 2001).

5.8 Conclusions and Outlook

Macrostomum lignano exhibits some appealing characteristics that qualifies this animal as an attractive model organism to study stem cells in flatworms. The exceptional amenability of the neoblast system renders *M. lignano* as a valuable addition to existing flatworm models. The development of additional methodological tools such as e.g. transgenic animals or further EST or genome sequencing might open new avenues to challenge stem cell related problems. However, progress using molecular tools must be accompanied by corresponding morphological studies. The determination and differentiation of neoblasts to build organs can be followed on the cellular level during postembryonic development and regeneration. The influence of regeneration or starvation on aging can be addressed. A detailed analysis of cell proliferation, migration and differentiation by applying e.g. double labeling of neoblasts with iodo- and chlorodeoxyuridine will yield important insights into the function of the *M. lignano* stem cell system the research that we have performed has demonstrated the usability of *M. lignano* in different research contexts and the potential for interdisciplinary approaches. *M. lignano* is suitable to integrate molecular, organismal and ecological analyses to address fundamental biomedical questions.

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Stem cells: from Hydra to man

The stem cell system of the basal flatworm *Macrostomum lignano*

Abbreviations

BrdU	5'-bromo-2'-deoxyuridine
EST	Expressed Sequence Tag
S-phase	synthesis-phase of the cell cycle
rER	rough endoplasmic reticulum
G2	second gap-phase of the cell cycle
TUNEL	TdT-mediated dUTP-biotin nick end labelling
DNA	deoxyribonucleic acid
BLAST	Basic Local Alignment Search Tool
RNA	ribonucleic acid
PCNA	Proliferating Cell Nuclear Antigen
MCM	Minichromosome Maintenance
mRNA	messenger RNA
RNAi	RNA (ribonucleic acid) interference
$MgCl_2$	magnesium chloride

Chapter 6
Regeneration and Stem Cells in Ascidians

Stefano Tiozzo[1], Federico D. Brown[2,3], and Anthony W. De Tomaso[1,4*]

Abstract Understanding and utilizing the ability of stem cells to expand and differentiate into tissues and organs is a major goal of biomedical science. Ascidians are basal chordates (Tunicata) which offer unique opportunities to investigate the biology of stem cells. These marine organisms begin their life as a larva with a typical chordate body plan, including a notochord, dorsal hollow nerve tube and a striated musculature. After a swimming phase, the larvae settles and undergoes an extensive metamorphosis during which most of the chordate characteristics are resorbed, leaving a filter feeding sessile invertebrate adult. Due to its small size (in many species a larva consists of ca. 2,500 cells) and rapid development (a fertilized egg can complete development in less that 24 h in *Ciona* species), the study of ascidian larvae has a long history and continues to be an outstanding model for studying specification and differentiation events which occur during chordate embryogenesis. In comparison, the adult body plan is relatively unstudied at a molecular level, but several examples of extensive regeneration following surgical ablation of different tissues have been described. In addition, within this chordate subphylum two distinct adult body plans exist: solitary and colonial. Following larval metamorphosis, solitary species grow into an adult that can range from several millimeters to tens of centimeters in length. In addition, colonial species grow not by increasing in size, but by asexually propagating, eventually creating a colony of genetically identical individuals that can cover areas of several square meters.

[1] Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA 94305, USA

[2] Biology Department, Center for Developmental Biology and Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195, USA

[3] Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250, USA

[4] Department of Biological Sciences, Stanford University, Hopkins Marine Station, 120 Oceanview Blvd, Pacific Grove, CA 93950, USA

[*] Author for correspondence

Tel.: 831 655 6206, Fax: 831 3750793
E-mail: tdet@stanford.edu

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Thus colonial ascidians have two independent developmental pathways to create an identical adult body plan, and are the only chordates with this regenerative ability. Taken together, the ascidians represent an excellent system to study the biology of both embryonic and adult stem cells.

Keywords Ascidians, asexual development, coloniality, blastogenesis, regeneration

6.1 Introduction

All multicellular organisms originate from a small set of totipotent embryonic cells: descendants of the fertilized egg which have the ability to expand and differentiate into a structured body plan during embryogenesis. However, individuals are not static entities, and during normal growth and aging, or after injury, differentiated cells, tissues and organs must be replenished and regenerated. In adults, this process of regeneration is thought to be carried out by stem or progenitor cells which retain the capacity to proliferate and differentiate throughout the lifespan of the individual. However the nature of these cells and the molecular mechanisms which control these processes are not well-understood. Furthermore, the degree to which different organisms can regenerate tissues and organs is not a conserved feature throughout evolution: a salamander can regenerate an amputated limb, but a human cannot. Why is there a diversity of regenerative potential, and what cellular and molecular mechanisms underlie these differences?

Ascidians are a class of marine organisms in the chordate subphylum Tunicata and represent modern-day descendants of the chordate ancestor (Zeng et al., 2006). In addition to this close phylogenetic relationship to the vertebrates, the ascidians have had a long and distinguished history as models for both embryonic and regenerative studies (Satoh, 1994). For example, several species have played key roles in the study of early development. Fecund individuals give rise to large numbers of gametes, which can be fertilized and will develop rapidly in seawater. Embryos are often transparent, and in many species have specialized cytoplasm which is differentially pigmented, allowing visual tracking during cell divisions (Swalla et al., 2004). These descriptions coupled to experimental manipulations of the embryo eventually led to the development of the first complete cell fate map. Moreover, it was found that segregation of this differently pigmented cytoplasm correlated to cell fate, leading to the concept that ascidian development was mosaic, i.e., cell fate is autonomous and directed by segregation of maternal determinants which control differentiation decisions (Conklin, 1905). These observations have stood the test of time, even though it is clear that development is not exclusively autonomous and may require interaction between neighboring cells. Subsequent analysis with modern lineage tracers in different species of solitary ascidians (Nishida, 1987; Nishida and Satoh, 1983, 1985, 1989; Zalokar and Sardet, 1984) confirmed most of Conklin's early results and have strengthened the idea that ascidians contain highly invariant lineage fates and determinate development. Insights into ascidian development have

accrued rapidly in the last decade due to the completion of whole genome sequence of several species, allowing high throughput and comparative genomic approaches to identify the gene regulatory networks underlying chordate embryogenesis (Imai et al., 2006). While this review is not focused on cell lineage decisions which occur during early development, the concept of autonomous differentiation is intriguing in context of regeneration in adult ascidians, the main theme of this chapter.

Following embryonic development, the tadpole larva will undergo a species-specific motile period to find a suitable area for settlement. It then undergoes a dramatic metamorphosis during which most of the chordate characteristics are resorbed, and a sessile, invertebrate adult body develops, called an *oozooid*. The adult body plan is compartmentalized and structured, and contains a pharynx, gut, heart, both a central and peripheral nervous system, a primitive endocrine system, and a complex mixture of blood cell types. At this point two divergent life histories can take place: the majority of ascidian species are solitary, and the oozooid will increase in size, become sexually mature and undergo the rest of its life cycle; in contrast, a number of species are colonial, and in this case growth of the genotype is not accomplished by the oozooid increasing in size, but rather by an asexual developmental program (budding), whereby entire new bodies, including all somatic and germline tissues, develop in a process called blastogenesis. This eventually results in a colony of genetically identical individuals, called blastozooids. They are embedded in a common extracellular, carbohydrate-derived matrix, called the tunic, and in some species also linked by an extracorporeal vasculature. Although physically linked within the colony, each zooid is an independent entity, and, in some species, can be separated from the rest of the colony and will continue to grow. Thus colonial ascidians have evolved two independent developmental pathways that can produce a nearly identical body plan both sexually, via embryogenesis/metamorphosis, and asexually, via blastogenesis.

Ascidian species are classified into three suborders (Aplousobranchia, Phlebobranchia, and Stolidobranchia) in which both solitary and colonial forms exist, suggesting that transitions between solitary and colonial life histories have occurred several times independently (Kott, 1981; Zeng et al., 2006). Moreover, as outlined below, the morphology of budding is not consistent among the colonial species, lending further evidence to multiple independent transitions to a colonial life history. A lack of robust phylogenies and fossil evidence makes it very difficult to infer the life history state of the tunicate ancestor. However there is one case where morphological and molecular evidence suggests a directional transition from a solitary ancestor into a colonial clade (Zeng et al., 2006), but how and why such a transition would have occurred is enigmatic. Are solitary ascidians, like salamanders, highly regenerative, maintaining pluri- or multipotent cells long into adult life that can regenerate structures consisting of multiple germ layers? Or was there a fundamental shift in the maintenance of potency, in the different cell lineages and their ability to contribute to development? Are the mechanisms underlying differentiation different in embryonic versus adult regenerative developmental pathways? If so, how could this have occurred? As will be discussed below, studies on regeneration in adult ascidians have a long history at a morphological level, but the underlying

cellular and molecular mechanisms are completely unknown. In contrast, due to the tractability of studying the embryo, early developmental pathways are becoming very well understood. Thus, these organisms provide a unique opportunity to study the relationship between embryonic and regenerative developmental pathways for every tissue and organ in the body.

6.2 Regeneration and Coloniality in Adult Ascidians

Adult ascidians have long been known to possess regenerative abilities, ranging from the replacement of experimentally ablated tissues in solitary species to the development of an entire body from a small cluster of undifferentiated cells in colonial species (Berrill, 1950). The solitary ascidian *Ciona intestinalis*, for example, has the potential to completely regenerate the neural complex after surgical removal (Bollner et al., 1992). Although slightly smaller, the regenerated complex resembles the original structure in all aspects, including the distribution of specific neuronal cells, as defined by expressed neurotransmitters (Bollner et al., 1992, 1995). This species can also completely regenerate a surgically ablated oral siphon, regenerating multiple tissues including epidermis, muscle fibers and neurons (Whittaker, 1975). In both cases, tissues from multiple germ layers are produced, suggesting the presence of multipotent stem cells. In the genus *Polycarpa*, the internal organs can be reconstituted starting from the peribranchial lining (Driesch, 1902) and the regeneration of a connective tissue such as the tunic has been described by Brien (1930), Peres (1948), Zaniolo and Trentin (1987), and Hirose et al (1995, 1997). However, it is in the colonial ascidians where the most remarkable regenerative plasticity has been documented.

After metamorphosis, colonial ascidians begin a lifelong, recurring budding process which eventually gives rise to a colony of genetically identical individuals (blastozooids, i.e. zooids which originate by asexual development, or blastogenesis) embedded in a common tunic, and in some species linked by a complex vascular system. Each blastozooid (herein called zooid) can replicate both sexually and asexually. During asexual propagation, adult zooids regenerate themselves, including all somatic tissues and the germline. As growth in colonial individuals is accomplished by budding, regeneration is an integral part of the life history. Mechanisms of asexual development differ substantially among colonial species, and can be classified based on the time of budding, the body region from where the bud initiates, the mode of bud formation and the type of multipotent cells contained in the buds (reviewed in Satoh, 1994 and Nakauchi, 1982; definitions of bolded terms are found in Glossary and outlined in Fig. 6.1).

Despite these morphological differences, there appears to be a common early stage of asexual development, which for descriptive purposes is called the triploblastic vesicle. This structure, which can be visually identified in nearly all budding types, is composed of a ball of three undifferentiated cell layers: ectoblast, mesoblast, and endoblast (Fig. 6.1, Brien, 1968). The ectoblast gives rise to the ectoderm of the

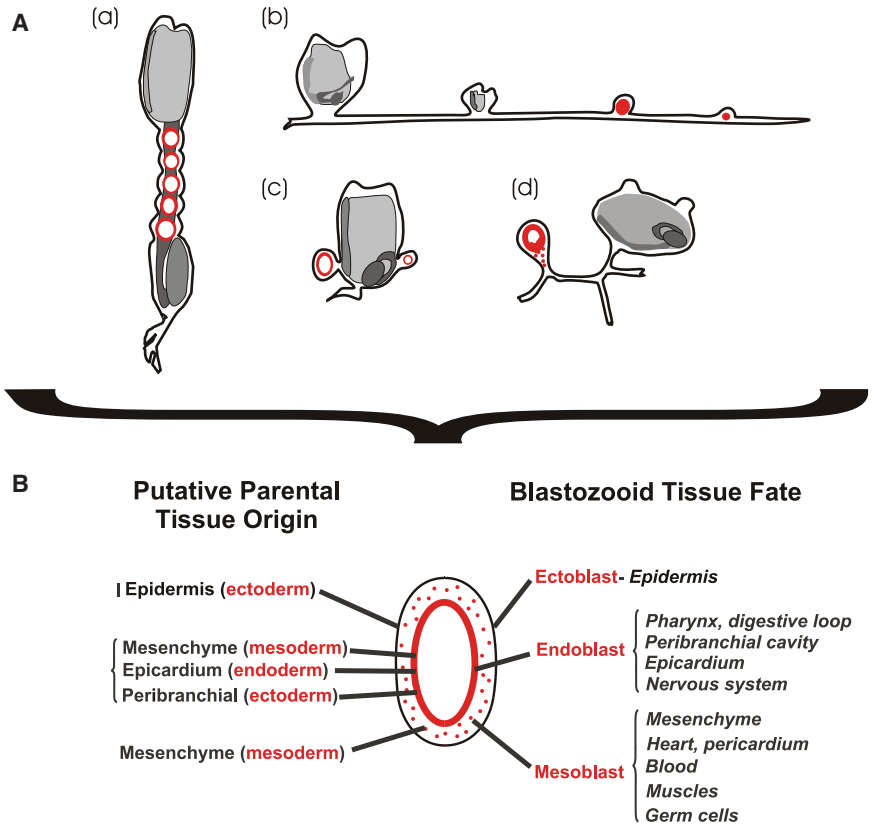

Fig. 6.1 Triploblastic buds are formed during colonial ascidian propagation. (**A**) Schematic localization of the triploblastic vesicle (red circles) in some types of budding: (a) abdominal strobilation, (b) stolonial budding, (c) palleal budding, (d) vascular budding. (**B**) Diagram of a triploblastic vesicle including putative parental origin and fates of each of the three bud layers during organogenesis of the blastozooid (from Manni and Burighel, 2006; Nakauchi, 1982; and Brien, 1968; modified)

blastozooid; the mesoblast is destined to differentiate into mesenchyme of the hemocoel, the cellular elements of the blood and the musculature; and the endoblast forms most of the internal organs: the pharynx, peripharyngeal chambers, digestive tract, epicardium, heart, and often the neurogranular system (Brien, 1968; Burighel and Cloney, 1997; Fig. 6.1). While in one sense the tripbloblastic vesicle could be compared to the early gastrula (i.e., after germ layer specification but prior to organogenesis), this is a generalization based only on visual description and there is no molecular or cellular evidence to support this comparison. Nevertheless, it is clear that all buds go through an early stage consisting of undifferentiated cell layers.

When the origins of these three bud layers is compared between colonial species, the ectoblast is usually derived from the epidermis of the parent; the mesoblast is usually inherited from the mesenchyme and blood cells of the parental zooid, while the endoblast origin varies among the species and the type of budding.

For example, in **stolonial budding** of some Polyclinidae and some Clavelinidae, it is derived from endodermal epicardial epithelium (Nakauchi, 1966); in Pherophoridae, i.e. *Perophora viridis* (Freeman, 1964) and *Perophora orientalis* (Fukumoto, 1971), species which also undergo stolonial budding, the endoblast originates from the **mesenchymal septum** within the **stolon** (Fig. 6.1). In Botryllidae and Styelidae: *Botryllus schlosseri*, *Botrylloides leachi*, *Botrylloides violaceus*, and *Polyandrocarpa misakiensis*, which develop by **palleal budding** and vascular budding, the endoblast originates from the peribranchial epithelium or cell aggregates originating from the blood respectively (Oka and Watanabe, 1957; Rinkevich et al., 2007; Fig. 6.1). In Polyclinidae like *Aplidium pallidum*, *Amoroucium yamazii*, and *Amoroucium constellatum*, which bud by **strobilation,** the endoblast originates from the digestive or epicardial tissue of an elongated abdominal section of the mother bud (Berrill, 1951; Fig. 6.1). However, despite these differences, one thing is clear: the early bud does not contain every adult cell type of the parental zooid, so progenitor cells must exist which can differentiate into the new tissues. This leads to one of the biggest mysteries of coloniality: what is the origin of potency in the early bud? Do long lived, pluripotent cells exist? Or else, are there populations of cells with restricted potential (e.g. hematopoietic/neural stem cells), or can certain differentiated cells de- or transdifferentiate?

To approach these questions, Hirschler (1914) cut individuals of *Ciona intestinalis* in half and observed that the part containing the epicardial (endodermal derived) tissue is capable of regenerating the whole organism. Several years later, Huxley (1921) performed similar experiments in the species *Perophora viridis*, analyzing budding under conditions of stress. All these authors referred to the regenerative process as 'dedifferentiation' of presumably preexisting somatic cells, however they were not able to characterize the modifications occurring at a cellular level, i.e. proliferation and differentiation. In contrast, Freeman (1964) used prospective isolation techniques to demonstrate that a subset of small, undifferentiated cells in the blood, called lymphocyte-like cells, could restore budding in irradiated colonies of *Pheropora viridis*, suggesting they were pluripotent stem cells. Initial experiments established that budding could be inhibited or blocked in a dose-dependent manner by gamma irradiation, which would induce breaks in the DNA, affecting cells which are rapidly dividing. However, budding could be rescued by the injection of unirradiated blood cells back into the irradiated colony. By separating the blood cells into 8 morphological groups, and injecting each purified population to assay its ability to rescue budding; only the 'lymphocyte' population of cells was able to rescue budding in the irradiated colony (Freeman, 1964). This was the first experiment to suggest that a single group of cells was responsible for budding, although since no lineage marking was done in the new buds, the mechanism of rescue remains unknown.

The pattern of cell proliferation in *Polyandrocarpa misakiensis*, a species that propagates by stolonial budding and palleal budding, also supported the idea of blood-based progenitors.Kawamura et al. (1988) found that a small population of haemocytes would incorporate [^{3}H]thymidine. These labeled cells aggregated in the inner epithelium of the double-walled bud vesicle and contributed to the development

of the bud. In a subsequent study, Suzuki et al. (1990) isolated a calcium-dependent, galactose-binding lectin expressed in the inner epithelium of the double-vesicle during bud formation. At the beginning of morphogenesis, the lectin was secreted into the mesenchymal space, becoming part of the extracellular matrix, where it appeared to direct the migration of a subset of proliferating haemocytes to the epithelium of the inner vesicle, suggesting a role in bud morphogenesis (Kawamura et al., 1991). These studies suggested that proliferating cells are present in the haemolymph and contribute to development of the zooid. However the classification of blood cells was based only on morphological differences, and molecular markers that recognize particular subpopulations of undifferentiated blood cells are required to further delineate these populations.

Laird and Weissman (2004) demonstrated in *Botryllus schlosseri* that telomerase activity is developmentally regulated. Telomerase is a critical component of self-renewal and maintains chromosomal integrity in long-lived stem cells. In most organisms, telomerase activity is high in undifferentiated cells, and activity progressively decreases as differentiation occurs, falling to basal rates in terminally differentiated lineages (Forsyth et al., 2002; Holt and Shay, 1999). These same kinetics were recapitulated during the 2-week budding cycle in *Botryllus schlosseri*: the highest level of telomerase activity is detected in the initial triploblastic vesicle and constantly decreased at each subsequent stage of budding. This suggests that cells with high self-renewal potential are involved in initiation of budding, but these cells subsequently expanded, committed, and terminally differentiated during development. The relatively rapid kinetics of telomerase activity (24 h) suggest that either a circulating population of stem cells migrate and cluster in the primordial bud, or conversely that a resident subset of quiescent cells becomes activated during the formation of the triploblastic vesicle.

As discussed below, the number of new buds which can develop off a single zooid is variable, and can be experimentally manipulated, thus the source of progenitors which give rise to various tissues in the bud is not clear. However, in some species it has been shown that lineage-marked blood based progenitors can give rise to the germline and some somatic tissues of the new bud (see Section 6.3). Interestingly, recent reports from several laboratories have shown the expression of two precursor cell specific markers in subsets of circulatory cells in the blood of Botryllidae. First, the germline specific transcript *vasa* has been detected in the circulating cells of *Botryllus schlosseri,* and *Botrylloides violaceous,* in addition to the putative expression in spermatogonia, and in the oocytes of zooids (Brown and Swalla, 2007; Brown et al. *in preparation*). Second, unpublished data from our lab have shown that *Sox2*, a transcription factor thought to maintain neural progenitor, stem cells, is expressed in scattered blood cells in *Botryllus schlosseri* (Tiozzo et al. *in preparation*).

The hypothesis that blood is the source of pluripotent cells is further supported via the study of an alternative budding pathway in the Botryllidae. In addition to the normal palleal budding, species in this family can also asexually develop in the vasculature, in a process called vascular budding (Oka and Watanabe, 1957; Berrill, 1951; Sabbadin et al., 1975). The initial vascular bud consists mainly of an aggregation of blood cells with lymphocyte like characteristics, which proliferate

and eventually form a round mono-layered epithelium that resembles the endoblast of palleal buds (Rinkevich et al., 2007). The ectoblast of the vascular bud appears to be derived from the ectodermal vasculature surrounding this initial endoblastic sphere (Oka and Watanabe, 1957), and development of a new zooid continues from this triploblastic vesicle, morphologically analogous to palleal budding. In some species, vascular budding occurs concurrently with palleal budding (*Botryllus primigenus* and *Botryllus delicates*; Oka and Watanabe, 1957; Okuyama and Saito, 2002), however, in other species it is initiated in the absence of palleal budding when the colony recovers from hibernation or aestivation (Rinkevich et al., 1995). In addition, vascular budding can also be initiated via experimental surgery, even in species where vascular budding does not normally occur (Voskoboynik et al., 2007; Rinkevich et al., 2007). For example, in *Botryllus schlosseri*, it has been found that if all the zooids are removed from a colony, leaving only the tunic, circulatory cells, and the peripheral vasculature (ectoderm), vascular budding will occur (Watkins, 1958; Sabbadin et al., 1975). Usually only 1–2 zooids will grow from the colonies, and, interestingly, these initial vascular buds often appear abnormal, with skewed axes and some significant heterochronies. However, they immediately begin palleal budding, and after several asexual cycles the zooids regain their proper form and continue to bud (Voskoboynik et al., 2007). Thus unlike other botryllid tunicates, vascular budding in *Botryllus schlosseri* is an induced phenomenon that requires the removal of zooids and buds, and the entire colony can be regenerated without preexisting zooids. This implies the presence of circulating multipotent or pluripotent cells that can migrate to other regions within the colony, find or form their own suitable niches and initiate budding.

In contrast, asexual propagation/regeneration in ascidians has also been attributed to Morgan's concept of **morphallaxis**, where somatic cells at the region of budding de-differentiate and regain pluripotency (Morgan, 1901; Brien, 1968). Morphallaxis can be contrasted with **epimorphosis**, which is characterized by a much greater relative degree of cellular proliferation and blastema formation prior to differentiation. Thus, in morphallaxis the majority of the regeneration comes from reorganization or exchange without much cellular movement, while in epimorphosis the majority of the regeneration comes from proliferation, formation of the blastema, then cellular differentiation. As outlined above, new tissues in the bud are formed from folding of specific epithelial layers (Berrill, 1950; Nakauchi, 1982), and the number of buds can be variable depending on the health of the colony. This could be interpreted as a de-differentiation process that initiates budding. Observations on bud development in *Polyandrocarpa misakiensis*, support this hypothesis. In this species, most of the new tissues are formed from folding of the atrial epithelium (Kawamura et al., 2008, 2008); Fujiwara and Kawamura (1992) found that Pae1, a tissue specific antigen expressed in the adult atrial epithelial cells, disappears selectively from the areas where the atrial epithelium forms organ rudiments during the asexual developmental program. The same epithelium, consisting of squamous cells that contained pigment granules, has also been characterized by AP (alkaline-phosphatase) expressed on the apical cell surface, which selectively disappears from the epithelium, and reappears in the digestive tract during gut formation in the new bud (Kawamura and Fujiwara,

1994). These regions in which the bud show high mitotic activity, are coupled with a thickening of the epithelium and an accumulation of a large amount of RNA. These observations show a change in the state of differentiation, suggesting a transdifferentiation-like process as strategy for organ formation in *Polyandrocarpa misakiensis* (Kawamura and Fujiwara, 1994; Kawamura et al., 2008).

In summary, it is not clear how many different pathways are involved in blastogenetic development in the Tunicata. Moreover, without adequate cell lineage tracing it is hard to resolve if the plasticity of budding is due to niche formation/pluripotent stem cell homing, or de-differentiation of pre-existing somatic cells, or both processes simultaneously. These hypotheses have already been pointed out by Kawamura and Fujiwara (1995) and earlier by Wolpert (1969) in his source-sink model of positional information theory. As illustrated in Fig. 6.2, a small part of the body may not contain the entire repertoire of cells required to regenerate all the tissues of the parent. In a 'strictly morphallactic' model (Model I), one or more populations of the inherited

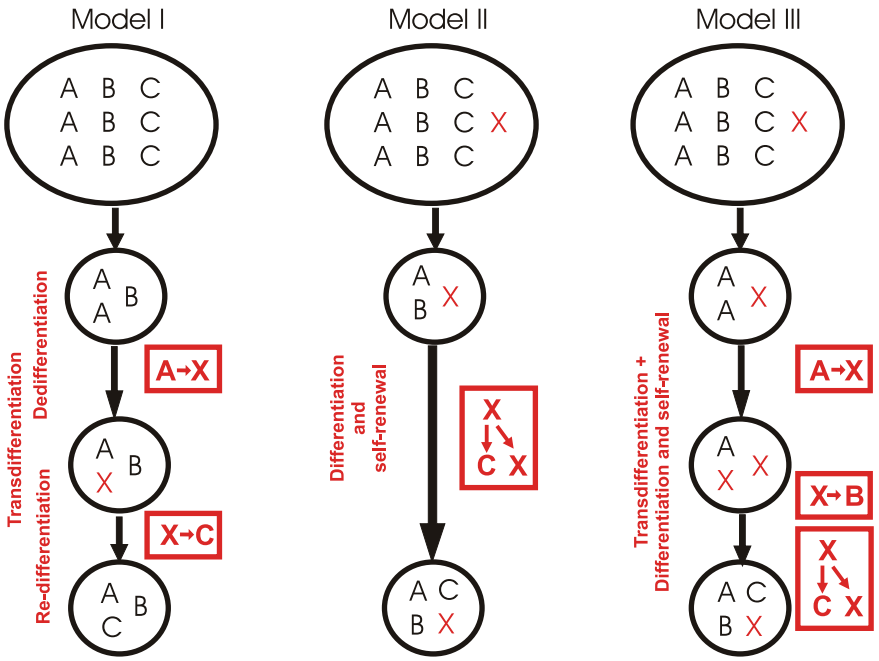

Fig. 6.2 Three possible models of cell contribution during budding and regeneration. We assume an animal consists of three or four sort of cells 'A', 'B', 'C' and 'X'. When a portion of the animal is isolated from the parent, it is composed by only part of the original set of cells, and it still has the ability to reconstruct the complete body. This regeneration may occur by a 'strictly morphallatic model' (left), where a multipotent cell A transdifferentiates into C; by differentiation and self renewal (center), where undifferentiated multipotent or pluripotent stem cells 'X' are the source for any lacking cell types, in this case cell type C; or by a combination of both transdifferentiation, differentiation and self-renewal (right), where multipotent and pluripotent stem cell precursors (X) co-occur together with multipotent cells/tissues able to go through transdifferentiation (A to C)

cells (A and B) could dedifferentiate (into X), which in turn can redifferentiate into the missing population of cells (C). Alternatively, in a 'stem cell model' (Model II) a source of stem cells could pass to the bud, self renew (into another X) and differentiate into 'C'. A combination of both processes, morphallaxis and epimorphosis may explain not only the regenerative power linked to the life history of colonial ascidians, but also the regenerative abilities of solitary species, such as regeneration of the central nervous system and siphons in *Ciona intestinalis* (Bollner et al., 1995).

6.3 Budding and Regeneration Niches: Another Level of Tissue Plasticity

The development of buds is often affected by conditions in the colony: for example, in healthy colonies multiple buds can grow simultaneously, while under conditions of stress budding can be halted or greatly reduced (Sabbadin, 1969). In addition, it appears that a number of colony-wide factors can also affect asexual development in a poorly understood process of communication between the colony and new buds. In *Botryllus schlosseri*, this cross-talk is seen in three experimentally inducible conditions: surgical manipulations of buds and zooids (Lauzon et al., 2002), *situ inversus viscerum* (Sabbadin et al., 1975), and inducible angiogenesis (Nyholm et al., 2006; Tiozzo et al., 2008). As outlined below, these processes suggest that epigenetic clues from the environment, i.e. pre-existing tissues, may drive patterning during budding, pointing to higher order regulation to these regenerative processes, or, in simpler terms, a niche which can be manipulated by conditions in the colony.

For example, microsurgical analyses have been used to investigate the interplay between the buds and zooids during the blastogenic cycle (Lauzon et al., 2002). In *Botryllus schlosseri*, each zooid has a 3 week life cycle, after which it dies, is resorbed, and replaced by a newly developing bud. It has been observed that the week-long lifespan of the adult feeding zooid was not altered by removal of all the buds in a colony, thus the lifespan of an individual zooid appears to be an intrinsic process. However, if the buds show delayed development, the adult zooids will die early, a phenomenon which is interpreted as a colony-wide transfer of energy and resources from adult to bud in order to maintain colony homeostasis.

In a related set of experiments, all the zooids were removed at certain stage of the blastogenetic stage, and it was found that this inhibited the growth rate and final size of the buds, presumably due to the lack of resource exchange between asexual generations. Interestingly, if all the buds, except one, are removed from a colony at the same stage, this single remaining bud resorbs all of the material of the colony, becoming a 'superbud', increasing 10–20X in size when compared to a normal bud (Lauzon et al., 2002). This is accomplished by hyperplasia, and this single bud is functional and can begin budding itself, eventually regenerating the entire colony. This demonstrates remarkable flexibility to bud growth, and supports the hypothesis that the allometry was derived from mutual interaction among the zooids (Nakaya et al., 2003). Moreover, the superbud can support budding of 10–20 new buds, demonstrating a plasticity to bud initiation and development.

Another fascinating example of this cross-talk is a manipulable asymmetry of the new bud. Normal zooids exhibit a bilateral asymmetry, where the heart is located on the right side of the organism, and the digestive tract is on the left side. 'Reverse' zooids, with the heart on the left and digestive track on the right, known as *situs inversus viscerum*, can be induced experimentally in several ways (Sabbadin, 1956; Oda and Watanabe, 1981, 1982; Kawamura and Watanabe, 1983), but appear to be the result of isolating the bud from the rest of the colony via a surgical manipulation. Interestingly, this reversed state is transmitted and maintained in the following asexual generations from that individual, but not in sexually derived offspring. Remarkably, when the colony is stimulated to propagate via vascular budding, all asexual progeny developed with the exact same asymmetrical state of the zooids in the colony they were derived from. In other words, if vascular budding was initiated from a colony in complete *situs inversus viscerum*, the regenerated colony stayed in situs *inversus viscerum* (Sabbadin et al., 1975). In summary, the bilateral asymmetry appears to be an epigenetic, asexually transmittable characteristic which is imprinted on the newly forming bud not just from the preexisting zooid but also from some sort of environmental signal present in the tunic and/or in the blood stream.

Finally, it has been observed that, when the peripheral circulatory system is surgically removed, it completely regenerates within 24–48 h (Tiozzo et al., 2008). The mechanism of vascular regeneration is not quite understood but a homolog of the Vascular Endothelial Growth Factor receptor (VEGFR) plays a key role in angiogenesis. Depleting the peripheral vasculature and inhibiting its regeneration via silencing VEGFR by siRNA, affected the organization of the newly developed zooids in the colony without altering the morphogenesis of the zooid itself, suggesting a role of VEGFR in structural support for the zooids within the tunic of the colony. This phenotype indicates that the peripheral vasculature may provide spatial information that functions to mechanically drive or orient the zooids after each blastogenetic cycle, and in the long term may model the shape of the colony.

Taken together, these manipulable processes provide a unique experimental system to look at the molecular mechanisms underlying patterning processes during asexual regeneration and suggests, at least in *Botryllus schlosseri*, a synergy between the role of multipotent/pluripotent cells and positional information provided from preexisting structures. These signals can drive patterning processes during morphogenesis and further affect the spatial organization of the entire colony. In other words, it appears that niches for budding and regeneration exist and contain both physical and chemical cues that in turn can be manipulated.

6.4 Self/Non-self Recognition and Parasitic Stem Cells

The source of multipotent cells which contribute to asexual development is still unclear. In addition, coloniality has arisen independently within the ascidian lineage multiple times, thus the mechanisms which create or maintain stem cells may not be evolutionarily conserved. Experiments in the botryllid ascidians provide an interesting case study on the source of somatic and germ line tissues, and began

with experiments on two disparate phenomena: a naturally occurring self/non-self reaction which occurs at the vasculature of two individuals, and conflicting interpretations from experiments designed to identify the source of the germline in newly developing buds.

Growth in colonial ascidians is due to budding, not growth of the newly metamorphosed individual, and is accompanied by an expansion over the substrate the colony is living on. This often results in a situation where two individuals grow into proximity, and the interaction leads to an allorecognition event, first described in Bancroft (1903), at terminal projections of the extracorporeal vasculature, called ampullae. Two outcomes can result from this interaction: either the ampullae will fuse together and form a single chimeric colony with a common vasculature, or they will reject each other, a process which ranges in morphology from a vigorous blood-based inflammatory reaction to a nearly imperceptible separation of the two individuals, but leading to the same outcome: the prevention of blood transfer between the individuals (reviewed in Saito et al., 1994). In all botryllid species described thus far, this reaction is controlled by a single, highly polymorphic locus with the following rules: two colonies will fuse if they share one or both alleles at this locus while those who share neither allele will reject each other, preventing blood transfer. The allorecognition locus is one of the most polymorphic ever described, effectively restricting fusion to kin, and begs the question as why a system like this would have evolved. The likely answer came from a completely different set of studies: the source of the germline in newly developing buds.

In a seminal paper describing the morphology of budding in *Botryllus schlosseri*, Berrill (1951), suggested that the germline developed *in situ* within the bud itself. In contrast, studies on a congenic species, *B. primigenus* (Mukai and Watanabe, 1976) suggested that germline maturation took several asexual cycles, with each generation contributing new progenitors during bud development which are later transferred to the subsequent bud through the common vasculature, and so on until development was completed. In an elegant series of experiments, Sabbadin and Zaniolo (1979) made use of the allorecognition reaction to approach this question, and made a fascinating discovery. They hypothesized that if germ cell progenitors did transfer between buds via a common vasculature, then they should also transfer between two fused individuals. Thus these investigators fused genetically disparate individuals (defined by different inherited pigment types) that shared a common fusibility allele and allowed them to interact for 2–7 days, after which they were surgically separated. At that point the genetic source of the germline was determined in each individual over time. It was found that germ cells had indeed transferred, and that colonies harbored the fusing partners' germline (both, eggs and sperm). However, in some pairs of colonies, complete germ cell replacement was found, and only one genotype was represented in the gametes (both male and female) of both colonies, and this situation remained constant for 15 weeks after separation, the length of the observations. This result suggested that germline stem or progenitor cells were passed from one individual to another, and that these cells had the ability to mobilize and shuttle between the two fused individuals. Once transplanted, these germline progenitors could proliferate, differentiate and contribute to asexual development, often replacing the cells of the

host. In other words, if germline progenitors from two individuals were mixed in one body, they could compete for germline positions in newly developing buds, with one genotype often winning over another.

This process, called germ cell parasitism (GCP), was later found to be a repeatable trait (Stoner and Weissman, 1996). As discussed above, zooids within a colonial ascidian are often autonomous, and pieces of a colony can be separated and will continue to grow. Thus multiple independent experiments can be done on a single genotype. If naïve portions (called subclones, or ramets) of a single genotype were separated and fused independently, the GCP winner (or loser) was found to be consistent. For example, if genotype F out-competed and replaced the germline of genotype B in one chimera, genotype F always outcompeted genotype B when naïve subclones of the two genotypes were fused together: GCP is a repeatable process. It was found that the ability to parasitize the germline of another genotype was arranged in a transitive hierarchy: if colony F could out compete colony G, and colony G could out compete colony B, then colony F would always out compete colony B, such that F > G > B in the ability to parasitize the germline, thus GCP was predictable. Moreover, the winner of the pairwise comparisons always won when it was fused to both of the other genotypes. Thus different genotypes had differing abilities to seed the germline of the newly arising buds, and this situation was static for up to 6 months after fusion. These results suggested that GCP was a genetically determined trait (Stoner et al., 1999). In addition, there is also parasitism at the somatic level (somatic cell parasitism, or SCP), although it is not nearly as repeatable as GCP. Taken together, this suggests that a highly polymorphic allorecognition system evolved to restrict the potential of adult stem cells to transplant between individuals, which can then disproportionately contribute to asexual development.

The ability of long-lived stem cells to transplant and compete in another genotype has been hypothesized as the underlying reason for the ubiquity of allorecognition in the metazoa, as natural tissue interactions are common, particularly in marine invertebrates (Buss, 1982, 1987). However, the simple Mendelian genetic allorecognition system in *Botryllus schlosseri* and congeners is not found in all fusible colonial ascidians. For example, *Diplosoma listeranum*, wherein the zooids are not connected by a common circulation, will fuse together and form chimeras, and this is not dependent on relatedness (Bishop and Sommerfeldt, 1999). This has been interpreted to be the result of a lack of mechanisms to exchange stem cells, either because no common vasculature exists, or potentially the result of a different mechanism of budding, such as morphallaxis (discussed above), where progenitors would not necessarily be mobile.

6.5 Prospective Isolation of Adult Stem Cells

As described above, Freeman (1963) performed the first stem cell transplantation experiments in ascidians utilizing classic prospective isolation techniques also used to isolate mammalian hematopoietic stem cells: ablation of host stem cell activity by irradiation, followed by the transplantation of candidate cells and testing their

role in reconstitution. While the lymphocyte-like cells were the only cells which could restore the budding process, because they were not genetically distinguishable from the irradiated host, the exact role they played in asexual development is unknown. In *Botryllus schlosseri*, the existence of GCP and SCP made it clear that blood-based progenitors from one genotype could contribute to asexual development of different tissues and organs in another individual. This led to questions as to the nature of the cells responsible, specifically if there were either pluripotent or separate germline and somatic cell lineages, and the molecular basis of the parasitic phenotype. These questions require isolation and study of the cells themselves.

Initially, it was found that irradiation of colonies could block budding, but development could not be rescued irrespective of the number or type of cells injected (Laird et al., 2005). In *Botryllus schlosseri*, palleal budding is a continuous process, and the zooids are transient structures with a 3 week lifespan. Because budding initiates on a zooid which is itself still developing, the lack of rescue was interpreted to mean that a putative budding niche was not discrete, but a transient part of the pre-existing generation. However, the genetic component to stem cell parasitism allowed another approach to be taken. Reciprocal transplantation experiments using unconditioned individuals revealed that stem cells could contribute to asexual development, and moreover that they maintained their GCP or SCP phenotype. In other words, if cells were isolated from a pair of colonies with known GCP/SCP phenotypes during fusion, the transplanted cells recapitulated those outcomes: cells isolated from a GCP winner genotype and transplanted into a loser always contributed to and often parasitized the host, while cells isolated from a loser genotype did not contribute to development in a winner colony: stem cells could be isolated by phenotype. Interestingly, despite their role in GCP/SCP, these cells were not normally found in the vasculature, as transplantation of blood revealed little to no chimerism in the recipient. Limiting dilution analysis of cells isolated from the whole colony suggested that 1 cell in 500 had progenitor activity. These cells could be enriched via FACS using ALDH activity as a marker (as detected by the fluorescent reagent, BAAA; Laird et al., 2005) up to ca. 1/50 cells. In all experiments the transplanted cells showed both long-term reconstitution as well as self-renewal potential, and contributed for development for over 20 asexual generations. Initial single-cell transplantation experiments also suggested that separate lineages of germline and somatic cells existed, however, better lineage tracing will be required to resolve this observation (Laird et al., 2005). While these experiments are still ongoing, the fact that stem cell activity can be both prospectively isolated and analyzed by limiting dilution analysis can be interpreted as an argument against dedifferentiation in *Botryllus schlosseri*, although much remains to be done before solid conclusions can be made.

6.6 Perspectives

The rapid development of both solitary and colonial ascidians offers amenable model systems to study the molecular mechanisms which underlie cell fate decisions. For colonial ascidians, it is clear that multiple developmental pathways can give

rise to the same adult body plan (Manni and Burighel, 2006; Tiozzo et al., 2005). But the source of the pluripotent cells and their ability to regenerate are still unknown. The contrast of mosaic development during embryogenesis to the demonstrable plasticity of adult regeneration within and among species is perhaps the biggest mystery of all, as it is difficult to understand how such disparate mechanisms of differentiation could co-exist in the same organism. Nevertheless, the presence of high-throughput genomic and proteomic technologies and recent advances in creating transgenic individuals bode well for the future utilization of these organisms.

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Glossary

***Epimorphosis*:** regeneration in which substantial cell proliferation precedes differentiation.

***Mesenchymal septum*:** single-layered septum that vertically divides the stolonial cavity.

***Morphallaxis*:** the regeneration of a body part or tissue by means of structural or cellular reorganization with only limited production of new cells, and, in correlation with them, a dedifferentiation without modification of their initial histological orientation.

***Peribranchial Budding*:** the bud rudiment appears as thickening disc of the mantel, i.e. the peribranchial leaflet and the overlying epidermis, that fold, eventually forming a double vesicle that develop outside the parental zooid (Sabbadin et al., 1975).

***Stolon*:** tubular branch that usually sprouts from the basal side of the ascidian body. It is composed by an external tunic with scattered cells, a middle epithelioectodermal layer, and an inner discontinuous layer of mesenchyme that lines the stolonic cavity through which blood flows.

***Stolonial Budding*:** the bud forms close to the tip of the stolon, and incorporates a mesenchymal septal and some blood cells. Eventually the bud develops into a new zooids connected with the other zooids by the stolon (Nakauchi, 1982).

***Strobilation*:** it involves the isolation of parts of the animal by the constriction of the body wall epidermis. It is differently categorized based on the location of the constriction (i.e. abdomen strobilation, post-abdomen strobilation and abdomen/post-abdomen strobilation; Nakauchi, 1982).

Chapter 7
In Vitro Control of Organogenesis by ActivinA Treatment of Amphibian and Mouse Stem Cells

Makoto Asashima[1,2,3,*], Akira Kurisaki[1], and Tatsuo Michiue[1]

Abstract After identification of "organizer region" in amphibian embryos by Spemann-Mangold in 1930s, presence of limited number of factors had been proposed for fundamental embryonic patterning with mesoderm formation. However, these factors had remained unknown for a long time. In 1998, we have identified activin as the mesoderm-inducing factor. In this chapter, with various conditions of activin A treatment, we demonstrated *in vitro* induction of various organs from *Xenopus* undifferentiated ectodermal cells. These induction methods have been further extended to the differentiation of mouse embryonic stem cells *in vitro*. These model systems for the development of various differentiated cell types, tissues, and even whole organs provide excellent tools for the study of developmental processes.

Keywords Activin, organogenesis, ES cell, Xenopus, organizer

[1] Organ Development Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1, Higashi, Tsukuba-city, Ibaraki 305-8562 Japan

[2] Department of Life Sciences (Biology), Graduate School of Arts and Sciences, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan

[3] Organ Regeneration Project, International Cooperative Research Program, Japan Science and Technology Agency (JST), 4-1-8, Honcho, Kawaguchi-city, Saitama 332-0012, Japan

[*] Author for correspondence

1-1-1, Higashi, Tsukuba-city, Ibaraki 305-8562 JAPAN, Tel./Fax: + 81-29-861-2575
E-mail: asashi@bio.c.u-tokyo.ac.jp

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7.1 Control of Organogenesis In Vitro Using Animal Caps from *Xenopus* Embryos

7.1.1 Preface

The establishment of body patterning in embryogenesis is a complex process mediated by a multitude of different factors. In 1924, Spemann and Mangold identified the pigment-dense animal region on the dorsal side of the amphibian embryo which appeared just before gastrulation, which they showed could induce the formation of multiple tissue types (Spemann and Mangold, 1924). The identification of this "organizer region" showed: (i) cell fate is not determined before gastrulation, illustrated by the ability of the organizer to induce neural tissue in ventral regions of the embryo after transplantation; and (ii) that the organizer induces differentiation via intercellular signaling.

Mesoderm formation is a key event in embryonic development, and it was widely thought that, following the reasoning of Spemann and Mangold, that basic embryonic patterning, including mesoderm induction, may be mediated by a restricted set of signaling molecules. Candidate mesoderm inducers were first identified in the 1980's, and included the Heparin-binding growth factor bFGF and TGF-beta2 (Slack et al., 1987; Rosa et al., 1988), both of which could induce some mesoderm formation. However, these factors did not prove to be proper organizers, since they failed to induce dorsal mesodermal tissues such as notochord. In a pivotal experiment in 1989, the subunit of ActivinA, isolated from culture medium of K562 leukemia cells, was shown to drive differentiation of animal caps into dorsal mesodermal tissues including notochord (Murata et al., 1988; Asashima et al., 1989). Subsequently, ActivinA was shown to be able to induce formation of a variety of mesodermal tissues (Smith et al., 1990; Albano et al., 1990; Sokol et al., 1990; Asashima et al., 1990; Asashima et al., 1991a). The demonstration of ActivinA in developing *Xenopus* embryos strongly suggested that this molecule regulates mesoderm formation in vivo (Asashima et al., 1991b; Fukui et al., 1993). ActivinA consists of two inhibinβ_A subunits which form a homodimer. A total of 5 different inhibinβ subunits (inhibinβ_A-inhibinβ_E) have been identified, thus giving five members of the Activin family. In ActivinB, C, D and E, inhibinβ_A forms a heterodimer with inhibinβ_B, inhibinβ_c, inhibinβ_D or inhibinβ_E, respectively (reviewed in Asashima, 1994; Ariizumi and Asashima, 1995b).

7.1.2 The Animal Cap Assay Model of Mesoderm Induction

In *Xenopus*, ectodermal cells at mid blastula stage remain pluripotent. The work of Spemann and Mangold showed that the cell fate of presumptive ectoderm is regulated by the involuting mesoderm. Furthermore, the combination of the ectodermal and endodermal regions directs induction of mesoderm from

ectoderm. The *Xenopus* animal cap assay provides an excellent model of these developmental events.

At the blastula stage there is a large cavity (blastocoel) in the animal hemisphere and ectodermal tissues around the animal pole form the blastocoelic roof which is composed of a few layers of cells. This region, the animal cap, can be easily dissected from *Xenopus* embryos (Fig. 7.1B, C). This tissue become globular within 1 hour of dissection due to the differences in junctional tension between inner and outer cells, making it easy to manipulate in differentiation assays (Fig. 7.1D). Untreated animal caps retain their globular shape in culture for about 7 days, and then differentiate into atypical epidermis. However, when treated in culture by inducing factors, animal caps will differentiate in various tissue types, thus enabling the simple screening of candidate differentiation factors.

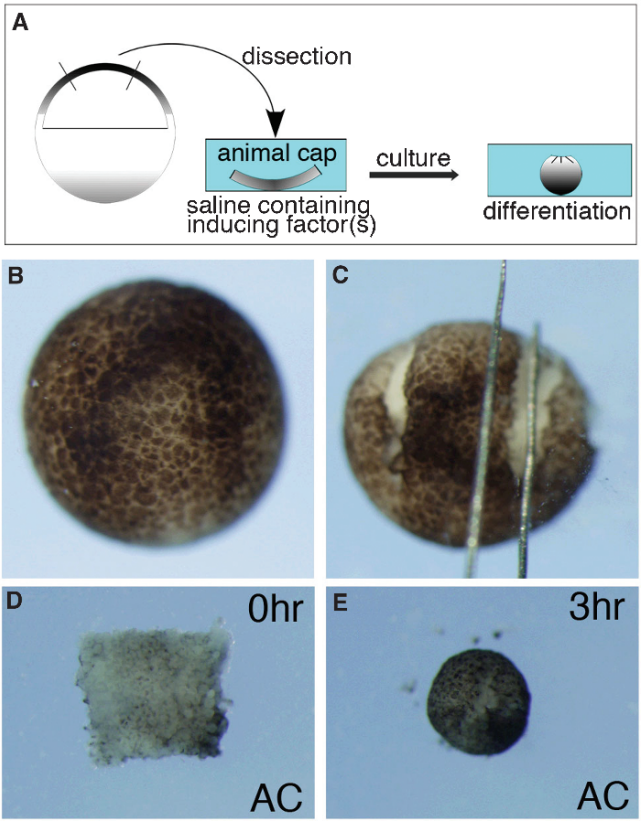

Fig. 7.1 Summary of animal cap assay (A). Mid-blastula embryo before dissection. The animal pole region was dissected away by tungsten needles (C). Animal cap is cultivated in saline. Just after dissection, the animal cap is sheet-like shaped (D), and after cultivation, it becomes globular in shape (E). Culture in saline containing candidate(s) growth factors enables evaluation of the activity of various factors on the animal cap cells

7.1.3 *Concentration-Dependent Induction of Various Organs by ActivinA*

As we outline in detail below, ActivinA can also induce ectodermal and endodermal tissue formation from animal caps, which is dependent upon the concentration of activinA Fig. 7.2. (Ariizumi et al., 1991). At very low concentrations (<0.5 ng/ml), ActivinA treatment drives cell fate of animal caps towards ectodermal lineages other than epidermis, at low concentrations (0.5–1.0 ng/ml) it induces ventral mesodermal tissues such as mesenchyme and hematopoietic cells, at higher concentrations it can induce mesodermal tissues such as muscle cell (5–10 ng/ml) and

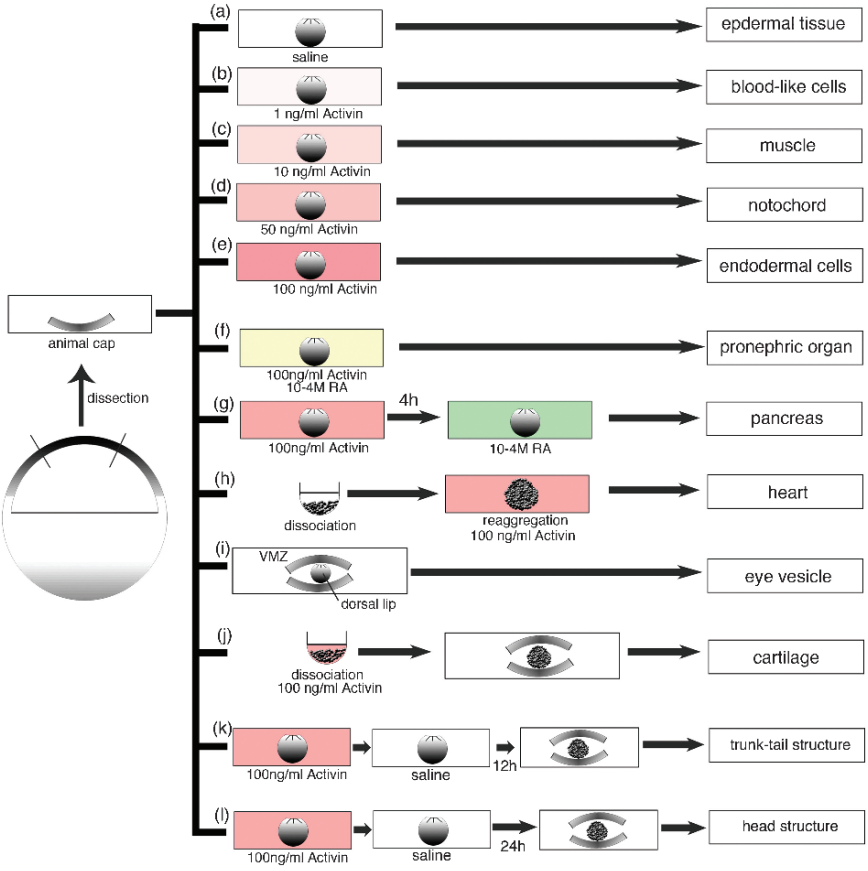

Fig. 7.2 Overview of organ induction from undifferentiated cells. When animal caps dissected at mid blastula stage were treated with various concentrations of ActivinA, several tissues differentiated (a–e). In addition, combination treatment with retinoic acid (RA) induces pronephros or pancreas (f, g). Furthermore, by dissociation and re-aggregation of animal caps or sandwiching of treated animal caps, heart (h), eye vesicle (i), cartilage (j), and parts of tadpole body (k, l) are induced

notochord (50–100 ng/ml), and at very high concentrations ActivinA may induce endodermal cell types (>100 ng/ml). The mechanism underlying these differential inductive effects according to ActivinA concentration may be via cells sensing the ligand concentration on ActivinA receptors by the absolute number of occupied receptors per cell (Dyson and Gurdon, 1998).

7.1.4 Organogenesis In Vitro from Animal Caps

In addition to its wide-ranging effects on cell fate, ActivinA can under certain conditions also induce formation of complete and functional organs from animal cap cells in vitro. By varying the concentrations of ActivinA and combining it with other inductive molecules such as retinoic acid, we have variously induced formation of functional pronephros, heart and eye, along with pancreatic tissue and cartilage.

7.1.4.1 Induction of Pronephros from Animal Cap Cells

In *Xenopus*, the pronephros, or primordial kidney, is derived from intermediate mesoderm. Treatment of animal caps with a combination of ActivinA at a concentration that can induce mesodermal cells (10 ng/ml) and retinoic acid (RA; 10^{-4} M) led to formation of pronephric tubules (Moriya et al., 1993). Histological analysis of these tubules confirmed that they expressed markers of intact pronephros, including Gremlin and c-ret, and markers of both tubules and ducts (Osafune et al., 2002). Furthermore, we transplanted these pronephric organs formed in vitro into embryos in which the presumptive kidney region had been removed by surgery. Surgically depleted embryos died within 10 days, whereas some of the transplanted embryos survived for up to 1 month, indicating that these artificial organs were functional in vivo (Chan et al., 1999).

7.1.4.2 Induction of Pancreatic Tissue

We also observed the formation of some pancreatic tissue when animal caps were co-treated with ActivinA and RA. While this occurred at very low rates, the efficiency of pancreas formation could be increased markedly if animal caps were treated with these two morphogens sequentially rather than simultaneously (Moriya et al., 2000). Treatment of animal caps with RA 3–5 hours after ActivinA increased the rate of pancreatic tissue formation to approximately 80%. Histology confirmed that these tissues contained acnus-like structures, and electron microscopy showed electron-dense granules characteristic of exocrine pancreatic tissues. These tissues also expressed pancreas-specific markers (Moriya et al., 2000). Using this approach, we were able to screen for, and identify, novel genes that may be involved in pancreas formation (Sogame et al., 2003).

7.1.4.3 Induction of Heart from Reaggregates of Dissociated Animal Cap Cells

By manipulating animal cap cells through a "dissociation-reaggregation" method, we have been able to induce the formation of more complex organ structures in vitro. Incubation of animal caps in calcium-free saline leads to dissociation of the caps into single cells. Subsequent incubation in normal culture medium that contains 100 ng/ml of ActivinA led to formation of heart that expressed specific cardiac markers such as Nkx2.5 and CarTI, and demonstrated spontaneous beating in culture within 3 days (Ariizumi et al., 2003). These cardiomyocyte bundles also had histological characteristics of heart tissue. Transplantation of this cardiac tissue into *Xenopus* embryos that had had the entire cardiac primordia surgically removed maintained viability of the embryo, which subsequently developed into an adult frog (Ariizumi et al., 2003). Moreover, when induced heart was transplanted into the abdominal region of a normal embryo, this embryo developed into an adult with two independent hearts. Interestingly, both hearts were smaller than that of normal frogs, but the total volume of the two hearts was estimated to be equivalent to the volume of one normal heart, suggesting that the heart induced from exogenous tissue was functioning as well as the endogeneous heart. We are also using this as a model to identify novel genes involved in heart formation (Ito et al., in preparation; Yamagishi et al., unpublished data).

7.1.4.4 Induction of an Eye Structure

A previous study showed that treatment with RA and cancanavalin A (ConA) induced anterior neural structures including forebrain and eye vesicle (Moriya et al., 1998). Another report showed that an eye structure that was morphologically similar to normal eye could be induced at high frequency by sandwiching the dorsal lip explants between sheets of untreated animal cap that contained and ventral marginal zone cells (Sedohara et al., 2003). When this eye formed in vitro was transplanted into a tadpole, the optic nerve was observed to extend to host forebrain, and the grafted eye was able to recognize external light stimuli, suggesting that the induced eye was, at least in part, functional. We have also subsequently successfully induced the formation of an intact eye vesicle by sandwiching two animal caps treated with different doses of ActivinA between untreated caps (Suzawa et al., unpublished data).

7.1.4.5 Induction of Cartilage

In a different kind of sandwiched explant, in which ActivinA-treated animal caps were dissociated and reaggregated, and then sandwiched between untreated ectoderm, cartilage-like cells were induced (Furue et al., 2002). These cells expressed the chondrocyte markers collagen type 2 and Cart-1.

7.1.5 Induction of Partial Tadpole Body Structures

In classical transplantation experiments with the Newt embryo, it was shown that dorsal lips dissected from early gastrula could induce head structures, whereas dorsal lips from mid gastrula could induce trunk-tail structures (Spemann, 1930). These observations suggest that the organizer region is composed of a "head" organizer and "trunk-tail" organizer. Subsequent molecular analyses clarified that formation of the head organizer requires several secreted factors including Cerberus, frzb and dickkopf (Bouwmeester et al., 1996; Wang et al., 1997; Leyns et al., 1997; Glinka et al., 1998). Interestingly, these results could be reproduced in animal caps treated with ActivinA (Ninomiya et al., 1998; Ariizumi and Asashima, 1995a). Animal caps that had been treated with 100 ng/ml ActivinA, cultured for an additional 12 hours in saline, and then sandwiched between two untreated animal caps differentiated into trunk-tail structures containing spinal cord, notochord and somite. However, when the post-treatment incubation time was extended for 24 hours, sandwiched explants differentiated into head structures with eye vesicles and cement glands. These results show that ActivinA can mediate organizer functions.

7.2 Regulation of In Vitro Differentiation of Mouse ES Cells

7.2.1 Differentiation of Tissues from Mouse Embryonic Stem Cells

Embryonic stem (ES) cells have generated enormous interest due to their capacity to self-renew and their potential for differentiation into many different cell types. ES cells are derived from the inner cell mass of the preimplantation mammalian embryos. They have been demonstrated to have the capacity to differentiate into almost any cell type when transplanted into embryos during early development (Evans and Kaufman, 1981; Martin, 1981; Doetschman et al., 1985). Mouse ES cells have been driven to differentiate in vitro into various cell lineages, including neurons, cardiomyocytes, pancreatic β cells, and hepatocytes (Loebel et al., 2003). Recently, we have successfully applied the organ induction protocols for *Xenopus* animal caps to mouse ES cells, leading to induction of pancreas, heart, and ciliated epithelial tissues.

7.2.2 Differentiation of Pancreatic Tissues

While a number of studies have induced the formation of pancreatic β cells from ES cells (Soria et al., 2000; Lumelsky et al., 2001; Hori et al., 2002; Shiroi et al., 2002; Blyszczuk et al., 2003; Kim et al., 2003; Leon-Quinto et al., 2004; Miyazaki et al., 2004), none have been able to induce pancreatic tissues or whole pancreas

from ES cells. We recently demonstrated that pancreas-like tissue containing all three components, endocrine cells, exocrine cells, and duct-like structures, could be successfully induced from mouse ES cells using a similar protocol for the induction of pancreas from the presumptive endoderm of *Xenopus* embryos (Moriya et al., 2000). We prepared multicellular aggregates of ES cells called embryoid bodies (EBs), which resemble early postimplantation embryos containing the three primary germ layers (Fig. 7.3B), and treated these in culture with activinA and RA. This approach induced differentiation of complex and functional pancreas that includes all endocrine (α, β, γ, and δ) cells, acinar cells, and pancreatic duct-like structures (Fig. 7.3C). In addition, we found that the ratio of exocrine to endocrine cells in the induced tissue was differentially regulated by the concentrations of activinA and RA (Nakanishi et al., 2007).

RT-PCR and quantitative PCR identified different contributions of activin and RA to the induction of pancreas. Both factors could suppress the expression of sonic hedgehog (Shh) alone or in combination, which has been shown to be essential for initiating differentiation of the dorsal pancreas in mouse and chicken embryos (Kim et al., 1997). RA was considered to be indispensable for the induction of gene expression of pancreatic markers amylase 2, insulin II, glucagon, Pdx-1, and Ppy. In contrast, activinA has been reported to induce endoderm differentiation from both human and mouse ES cells (Kubo et al., 2004; D'Amour et al., 2005; Yasunaga et al., 2005). The level of expression of the early endoderm development marker Sox17 was significantly elevated in the EBs treated with activinA, indicating that activinA had a role in endoderm induction in this system. Interestingly, low concentrations of activin (10 ng/ml) induced much higher expression of amylase 2 than that of untreated EBs, while at a higher activin concentration (25 ng/ml), the

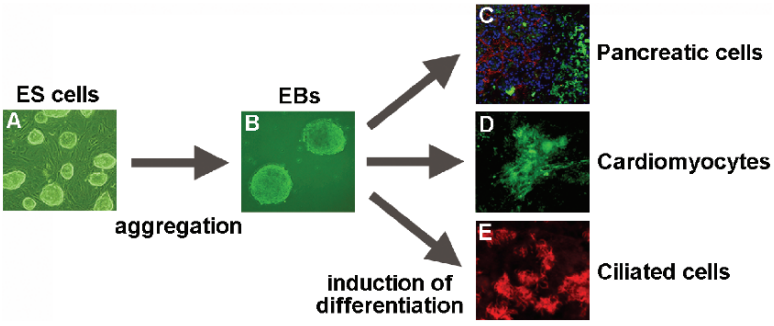

Fig. 7.3 Induction of differentiated tissues from mouse ES cells in vitro. (A) Mouse ES cells cultured on the mouse embryonic fibroblast feeder cells. (B) Multicellular aggregates, EBs, prepared from mouse ES cells. (C-E) Immunohistochemical staining of tissues that differentiated from mouse ES cells. (C) Pancreatic tissue induced by activin and RA was stained with anti-insulin C peptide antibody (green), anti-amylase antibody (red) and DAPI (40,6-diamidino-2-phenylindole) (blue). (D) Cardiomyocytes induced by BMP4 in the serum-free medium were stained with cardiac troponin T (cTnT) antibody (green). (E) Ciliated epithelial cells differentiated in serum-free medium were stained with anti-β-tubulin IV antibody (red)

expression of insulin was markedly increased in the EBs treated with both activinA and RA. These results suggested another possible role for activin in the differentiation and/or the proliferation of pancreatic endocrine and exocrine cells, when applied concomitant with RA. Thus, this relatively simple system for the induction of the differentiation of ES cells into pancreas-like tissue in vitro could provide a good model for analyzing the mechanisms of pancreatic development.

7.2.3 Differentiation of Cardiomyocytes

Cardiomyocytes are one cell type that can actually spontaneously differentiate from EBs, but this only occurs at a very low rate. There is currently no established method for the induction of cardiomyocytes from ES cells at high frequency. Some growth factors have been reported to promote the differentiation of ES cells into cardiomyocytes, and coculture with visceral endoderm-like cells or stromal cells has been demonstrated to improve efficiency of induction of cardiomyocytes from mouse and human ES cells (Johansson and Wiles, 1995; Wobus et al., 1997; Sachinidis et al., 2003; Honda et al., 2005).

With most experimental approaches used to date, the differentiation of cardiomyocytes was examined in the presence of serum, and therefore these approaches have difficulties for the clinical application because serum introduces a major risk of contamination with unknown viruses. Moreover, in vitro cardiomyocyte differentiation system with a clearly defined set of chemicals or growth factors could provide a simple model to study the regulatory mechanisms of cardiomyocyte development. However, under serum-free conditions, ES cell aggregates do not form a three germ layer-like structure leading to strong suppression of mesodermal expression (Johansson and Wiles, 1995; Watanabe et al., 2005). To develop a system for induction of cardiomyocytes under serum-free conditions, we attempted to adapt our *Xenopus* organ induction method.

We initially tested activin since this induced cardiomyocytes in *Xenopus* (Ariizumi et al., 2003), but at no concentration was it able to induce any cardiac differentiation of mouse ES cells. We then tested a related factor, bone morphogenetic protein (BMP), since BMP has been shown to induce ventral mesoderm in *Xenopus* laevis and mouse ES cells (Dale et al., 1992; Johansson and Wiles, 1995; Ng et al., 2005). We first prepared EBs, by forming cell aggregates with mouse ES cells, which were cultured in DMEM supplemented with a serum replacement (Knockout Serum Replacement, KSR), treated with BMP4 for 4 days, and then further cultured without BMP4. After 3 days of culture in the absence of BMP4 (differentiation day 7), there was the appearance of some spontaneously beating cardiomyocytes. The number of these cells increased with further culture, and the cells expressed a terminal differentiation marker, cardiac troponin T (cTnT) (Fig. 7.3D). RT-PCR analyses confirmed the specificity of this cardiomyocyte differentiation, identifying expression of mesodermal marker genes, such as Flk1, Pdgfrα and Pdgfrβ, followed by the transient induction of early mesodermal markers, brachyury and goosecoid.

7.2.4 N-Cadherin Is a Marker of Cardiomyocyte Progenitor Cells

One approach to improve the efficiency of induction of specific cell types in vitro is to sort cells by specific cell surface antigens. Flk1, also known as vascular endothelial growth factor receptor 2 (VEGFR2), is considered to be a cell surface marker for progenitors of cardiomyocytes (Motoike et al., 2003; Ema et al., 2006). Purified Flk1-positive cells have been previously demonstrated to differentiate into cardiomyocytes or hematopoietic cells in vitro (Nishikawa et al., 1998; Iida et al., 2005; Yamashita et al., 2005). However, another study has suggested that cardiomyocytes can arise from an Flk1-negative subpopulation (Kouskoff et al., 2005). N-cadherin, a glycoprotein that mediates cell adhesion, is expressed in cardiogenic mesoderm at various stages of mouse development (Radice et al., 1997). When we fractionated the cardiogenic progenitor cells induced with our method by FACS, N-cadherin-positive cells showed significantly higher levels of expression of the cardiogenic markers, Nkx2.5, Tbx5, and Isl1. Moreover, these cells had a significantly higher (7.9-fold) ability to differentiate into cardiomyocytes when they were differentiated on stromal cells for 6 days. Since N-cadherin signals are required for the maintenance of the structural integrity of the heart and the myofibril continuity across the plasma membrane (Luo and Radice, 2003) and regulate the expression of intercalated disc component such as connexin 43 through β-catenin or Rac1 (Matsuda et al., 2006), N-cadherin may be useful marker of a population of cells that will differentiate into cardiomyocytes.

7.2.5 RA-Dependent Differentiation of Cardiomyocytes

RA has also been suggested to regulate cardiomyocyte differentiation. Retinoid X receptors (RXRs) are important for the development and formation of the cardiovascular system in vivo. Null mutation of the mouse RXRα gene is lethal due to myocardial malformation (Kastner et al., 1994; Gruber et al., 1996), but mice deficient for specific RA receptors (RARα (Li et al., 1993; Lufkin et al., 1993), RARβ (Mendelsohn et al., 1994), and RARγ (Lohnes et al., 1993)) showed no obvious defects. Suppression of RAR signaling enhanced cardiomyocyte gene expression in zebrafish embryos (Keegan et al., 2005). Although these studies indicated that RA signaling is essential for cardiovascular development, it is still unclear how it works. Moreover, the spontaneous conversion of all-trans RA into 9-cis RA, and vice versa, makes it difficult to evaluate the specific functions of RA compounds (Pijnappel et al., 1993; Sucov and Evans, 1995). Therefore, we took advantage of RXR- or RAR-specific agonists or antagonists to monitor the effects of specific RA species, and analyzed the cardiomyocytes differentiation of mouse ES cells under the serum free conditions in vitro (Honda et al., 2005). The number of beating cardiomyocytes differentiated from mouse ES cells significantly increased following treatment with a synthetic compound, PA024, an RXR agonist. In contrast, when

EBs were treated with an RXR antagonist, PA452, the number of beating EBs was decreased in a dose-dependent manner. Our results suggest that RXR signaling regulates cardiomyocyte numbers during the differentiation of ES cells in vitro and probably in normal development.

7.2.6 Induction of Ciliated Epithelial Cells

We have also successfully induced development of ciliated epithelial cells from mouse ES cells under serum-free culture conditions (Nishimura et al., 2006). The cells with cilia-like motility were induced by floating culture of EBs in DMEM medium supplemented with 10% FBS for 3 days followed by the culture on a gelatin-coated dish in DMEM with 10% KSR for 2–3 weeks. Previous methods for inducing ciliated cells in vitro from embryonic or adult tissues involved culture of cells at an air-liquid interface (Coraux et al., 2005), which mimics the conditions of an adult trachea. But this is very different to the environment that ciliated cells normally differentiate in during normal development. We have developed a system for induction of ciliated cells from ES cells that more closely mimics the normal development of ciliated cells, and which provides a useful tool for studying the differentiation mechanisms of normal ciliated epithelial cells. The differentiated cells with our method expressed ciliary marker proteins such as β-tubulin IV (Fig. 7.3E), and a transcription factor essential to ciliogenesis, hepatocyte nuclear factor-3/forkhead homolog 4 (HFH-4). Electron microscopic observation revealed that the processed microtubules were arranged in the 9 + 2 structure that is the same specific alignment observed in normal ciliary microtubules. The cilia of these cells were actively beating at a frequency of 17–20 Hz, which is comparable to the ciliated cells of normal respiratory tract (approximately 20 Hz). The differentiated cells containing these ciliated cells expressed respiratory marker genes such as thyroid transcription factor-1 and surfactant protein-C, but there was no expression of Ovgp1, a marker gene for the oviduct. Thus, the ciliated epithelial cells induced in vitro with our protocol most closely represent those found in respiratory tissues such as trachea or lung in vivo, although we could not discount the possibility that they are ciliated cells of other organs, such as brain.

For the induction of ciliated cells, EBs must be cultured in a serum-free medium but not with a FBS-containing medium. We noted that BMPs also inhibit this differentiation. During lung bud extension in early embryonic development, cells expressing high levels of BMP4 differentiate into distal cells, whereas cells with low expression of BMP4 differentiate into proximal cells, including ciliated cells and clara cells (Weaver et al., 1999). Our result is consistent with this model in which BMP4 determines the distal-proximal polarity of respiratory tract.

The induction system we developed provides a useful means of studying differentiation mechanisms for ciliated cells and potentially for other respiratory cells in vitro. In addition, the system could be useful to analyze the effects of harmful substances on fetal ciliated epithelial cells.

7.2.7 Chromatin-Related Proteins Specifically Expressed in Pluripotent Mouse ES Cells

As described above, undifferentiated cells, such as amphibian animal caps and ES cells, respond to activin and other factors, and differentiate into various tissues. How these cells maintain their abilities to differentiate into many cell lineages is an interesting issue. Certain factors have been shown to be crucial for the maintenance of the undifferentiated state of mouse ES cells, including leukemia inhibitory factor (LIF), BMPs, Oct-3/4, Sox2, and Nanog (Pan and Thomson, 2007). However, the mechanisms of regulation of pluripotency are still not fully understood. To identify new proteins involved in the regulation of pluripotency, we performed a differential proteomic analysis of mouse ES cells using proteins extracted from undifferentiated and differentiated mouse ES cells. More than 50 proteins were identified as being specifically expressed in undifferentiated stem cells, including a number of chromatin-related proteins (Kurisaki et al., 2005). These chromatin-related proteins were highly expressed in pluripotent mouse ES cells, and were then dramatically downregulated during differentiation. Analysis with real-time RT-PCR revealed that enrichment of these proteins in pluripotent ES cells was regulated at the transcriptional level. These results suggest that specific chromatin-related proteins may be involved in maintaining the unique properties of pluripotent ES cells. Interestingly, our findings support the idea that chromatin dynamics is important for the regulation of stem cell differentiation and early lineage decisions during embryogenesis (Rasmussen, 2003). Furthermore, we demonstrated that the proteomic approach is an extremely powerful method to elucidate the mechanisms of pluripotency and differentiation process in the stem cells.

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Chapter 8
Melanocyte Stem Cells: As an Excellent Model to Study Stem Cell Biology

Masatake Osawa[1,*], Kiyotaka Hasegawa[1], Mariko Moriyama[1], and Shin-Ichi Nishikawa[2]

Abstract Elucidation of molecular mechanisms underlying stem cell regulation is of great importance for their clinical applications in regenerative medicine and cancer therapy. The function of stem cells is maintained by their specialized microenvironment, referred as the niche. Despite intensive studies of the stem cell niche, the molecular basis of stem cell regulation by the niche has still remained elusive. Since molecular interactions between stem cells and the niche can be analyzed only under *in vivo* conditions, one drawback that hampers stem cell research is the lack of an efficient *in vivo* assay system that allow to define an *in vivo* gene function for the regulation of stem cells. We have previously identified melanocyte stem cells (MSCs) in the mouse hair follicle, in which MSCs reside at a specific region of the hair follicle, termed as the lower permanent portion. MSCs offer an attractive model with which to study the molecular basis of stem cell regulation, because loss-of-function mutations in the genes responsible for MSC regulation are readily identifiable by a premature hair graying phenotype in mice. This implies the irresistible possibility that MSCs allows us to identify the genes involved in stem cell regulation by a phenotype-driven genetic screen in mice. Hence, we believe that MSC system is an excellent model to study stem cell biology.

Keywords Stem cells, melanocytes, melanoblasts, stem cell niche, Notch

[1] Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, MGH-East, Building 149, 13th Street, Charlestown, MA 02129, USA

[2] Center for Developmental Biology, 2-2-3 Minatojima, Minami-machi, Chuo-ku, Kobe 650-0047, Japan

[*] Author for correspondence

Tel: 1-617-726-4186, Fax: 1-617-726-4453
E-mail: masatake.osawa@cbrc2.mgh.harvard.edu

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8.1 Introduction

Stem cells play crucial roles in the maintenance of tissue homeostasis by replenishing cells comprising a tissue throughout a lifetime. Elucidation of molecular mechanisms underlying the regulation of stem cells is of great importance for both basic biology and their clinical applications in regenerative medicine and cancer therapy. Stem cells are thought to be regulated by their specialized microenvironment, referred as the stem cell niche (Li and Xie, 2005; Spradling et al., 2001; Watt and Hogan, 2000). Despite recent intensive studies of the stem cell niche, the exact molecular mechanisms underlying stem cell regulation by the niche have remained largely unclear especially in higher organisms such as mammals. This is in clear contrast to germline stem cells (GSCs) in the gonad of Drosophila and C. elegans, where the nature of cells comprising the niche and their role in the regulation of the stem cells have been well characterized at both cellular and molecular levels (Kiger et al., 2001; Spradling et al., 2001; Tulina and Matunis, 2001). One clear difference between these stem cell systems in Drosophila and C. elegans and those in mammals is the ability to identify both individual stem cells and the cells comprising the niche by their location and morphology. Hence, by combining the feasibility of genetic manipulation in these organisms, these GSC systems exert experimental advantages in elucidating the exact cellular and molecular interactions between stem cells and the niche (Spradling et al., 2001). By contrast, in mammals, it is often the case that stem cells comprise an extremely rare cell population within an entire tissue, and the surrounding microenvironment is composed of complicated anatomic structures. Under these situations, one drawback that hampers stem cell research in mammals is the difficulty in locating and manipulating individual stem cells and their niches. Therefore, development of a novel model system that enable us to identify and manipulate individual stem cells and the niche is highly anticipated to elucidate stem cell regulations by the niche in a mammalian tissue.

Melanocytes are specialized pigment cells that are responsible for hair and skin pigmentation. We previously identified stem cells of a melanocyte lineage (melanocyte stem cells: MSCs) in the mouse hair follicle (Nishimura et al., 2002). MSC system offers an ideal model system by which to study mammalian stem cells, as they are capable of localizing individual stem cells in the niche. This review summarizes recent advances in studies of MSCs in the hair follicle. We also discuss the advantage of MSC system to study molecular basis of stem cell maintenance by the niche.

8.2 Melanocyte Stem Cells in the Hair Follicle

Melanocytes are pigment cells responsible for pigmentation of animals. Melanocytes synthesize pigment melanin within a special organelle termed as melanosome where a number of melanocyte-specific enzymes involved in melanin biosynthesis are assembled, and thereby transfer pigment granules into adjacent keratinocytes to form pigmented skin or hairs (Lin and Fisher, 2007). These pigment granules

absorb ultraviolet (UV) radiation, and play an important role in protecting epidermal cells from UV-induced genotoxic stress, while the physiological role of hair pigmentation is less clear.

The hair follicle is a continuously regenerating system; two-thirds of lower hair follicle (transit portion) completely reforms itself over the hair cycle whereas the upper permanent follicle (permanent portion) is maintained throughout hair cycling (Stenn and Paus, 2001). In this regard, the hair follicle is an ideal model by which process of tissue regeneration is allowed to be analyzed under the physiological condition. The periodic hair cycle consists three distinct phases of hair follicle growth phase (anagen), followed by a regression phase (catagen), and a resting phase (telogen) (Stenn and Paus, 2001). Melanocytes appear at the onset of anagen phase in the hair matrix where they actively proliferate and differentiate into mature melanocytes, thereby transferring melanin granules into the hair shaft-forming keratinocytes to form pigmented hairs. Once terminally differentiated, these melanocytes are eliminated by apoptosis during catagen, and become absent in the telogen hair follicle until the next melnogenesis initiated in the subsequent anagen phase (Slominski et al., 2005). This regenerative cycle of melanogenesis is maintained by a small population of MSCs localizing at the lower permanent portion (LPP) of the hair follicle where individual stem cells are anatomically segregated from their differentiated progenies in the hair matrix (Fig. 8.1) (Nishimura et al.,

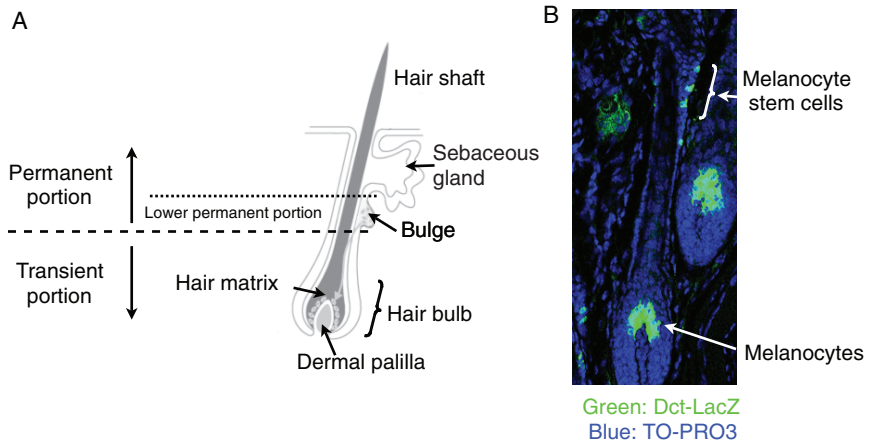

Fig. 8.1 Structure of the hair follicle and localization of melanocyte stem cells in the hair follicle. A Schematic representation of the mouse hair follicle. A hair follicle is divided into two portions: two-thirds of lower hair follicle (transit portion) completely reforms itself over the hair cycle whereas the upper permanent follicle (permanent portion) is maintained throughout hair cycling. Melanocyte stem cells reside in the lower permanent portion. B Localization of melanocytes in the anagen hair follicle. A skin section obtained from Dct-LaZ transgenic mouse, in which melanocytes are specifically marked with beta-galactosidase (LacZ), was stained with anti-LacZ antibody (shown in green) to visualize the cells in a melanocyte lineage in the hair follicle. In the hair follicle, most of the melanocytes localize in the hair matrix. Melanocyte stem cells are found in the lower permanent portion

2002). This feature of MSCs enables us to identify the individual stem cells based on their specific location (Fig. 8.1). Hence, as we have learnt from the example of GSCs in Drosophila and C. elegans, MSCs are advantageous to clarify the behavior of stem cells in the niche and to understand how stem cells are regulated by their surrounding microenvironment. Interestingly, MSCs reside in the LPP being scattered randomly without forming into cell clusters (Fig. 8.1). This reminds us of a possibility that the LPP itself serves a niche for the MSCs rather than depending on a specific niche cells present at the LPP.

8.3 Hair Graying and Melanocyte Stem Cells Maintenance

Melanocytes afford an advantageous model to understand the molecular bases of various cellular regulations, since genetic alterations involved in melanocyte regulations (i.e. in survival, proliferation, migration, differentiation or cell-cell interactions) are easily identifiable as coat color mutants in animals, which are frequently non-lethal and thus easy to select and maintain the mutant in a line. Indeed, currently more than 120 different loci have been identified as coat color mutants in mice (Bennett and Lamoreux, 2003). Although there are some exceptions, these mutants fall into roughly two distinct functional subgroups: one is the loci required for melanocyte development and the other is the loci essential for melanogenesis or melanocyte function. Mutations in the genes required for melanocyte development typically result in congenital patches of unpigmented hair and skin due to severe reduction of melanocytes, whereas alterations in the genes required for melanocyte function show variable degree of hypopigmentation or hyperpigmentation phenotypes without affecting number of melanocytes present in the skin (Bennett and Lamoreux, 2003).

In addition to these mutants, recent studies uncovered a novel class of pigmentation mutant mice that exhibit premature hair graying from the second hair cycle (Mak et al., 2006; Moriyama et al., 2006; Nishimura et al., 2005) (Fig. 8.2). Although hair graying represents the most common phenotype of human aging, it has remained largely unknown how aging impacts on hair pigmentation. As reactive radicals are continuously produced from the chemical reactions associated with melanin synthesis, one hypothesis that had been proposed to explain hair graying was that the continuous oxidative stress might promote apoptotic elimination of mature melanocytes in the hair matrix (Arck et al., 2006). However, recent studies of several mouse models of hair graying have revealed that hair graying stems from selective loss of MSCs rather than elimination of mature melanocytes (Mak et al., 2006; Nishimura et al., 2005). *Bcl2*-/- mice are born normal pigmentation but loose hair pigmentation in the second hair cycle (Fig. 8.2). Histologically, *Bcl2*-/- mice show dramatic elimination of MSCs at the LPP during the first anagen, while mature melanocytes remain in the hair matrix until the end of the first anagen (Mak et al., 2006; Nishimura et al., 2005). Similarly, loss of MSCs and its associated hair graying phenotype have been also reported in $Mitf^{vit/vit}$ and the mice in which Notch signaling is conditionally ablated in melanocyte lineage (Nishimura et al., 2005;

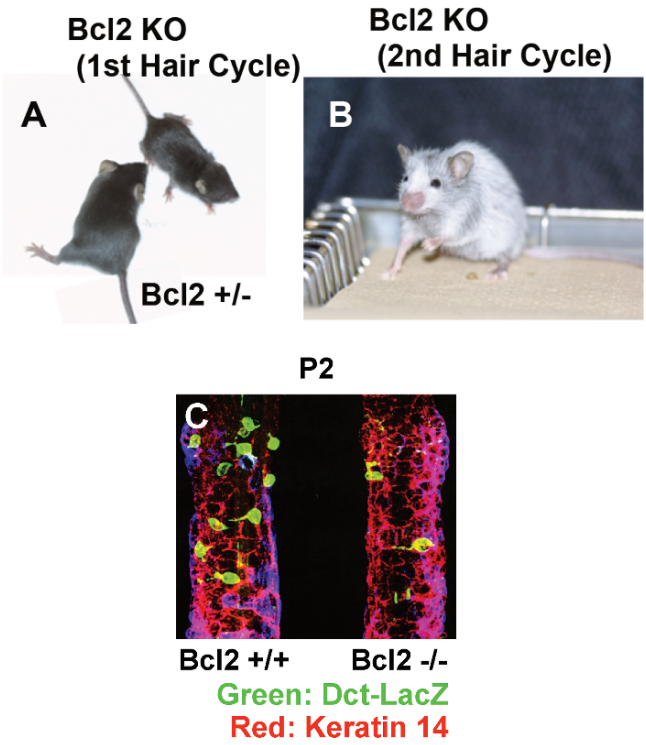

Fig. 8.2 Loss of melanocyte stem cells in Bcl2-/- mouse results in hair graying phenotype from the second hair cycle. A, B Coat phenotype of Bcl2-/- (Bcl2KO) mouse. Hair pigmentation of Bcl2KO mouse is normal in the first hair cycle (A), while in the second hair cycle Bcl2KO mouse exhibits premature hair graying phenotype (B). C Loss of melanocyte stem cells in the lower permanent portion of the hair follicle in Bcl2KO mouse. To mark melanocyte stem cells, Bcl2-/- mice were crossed with Dct-LacZ transgenic mice to obtain Bcl2-/-; Dct-LacZ mice. A single guard hair follicle was isolated from either wild type or Bcl2-/- mouse, and whole mount immunostaining was performed using anti-LacZ antibody to detect melanocyte stem cells in the lower permanent portion. Severe reduction of melanocyte stem cells is evident in the Bcl2-/- hair follicle. These data clearly indicate that a defect in the maintenance of melanocyte stem cells is easily identifiable by this typical hair graying phenotype

Quevedo and Holstein, 1992). Hence, it has been clearly demonstrated from these observations that hair graying is attributed to improper maintenance of MSCs. Given that loss of MSC results in hair graying from the second hair cycle, the loss-of-function of the genes responsible for the maintenance of MSCs could be readily identified by this typical hair graying phenotype. Thus, by integrating reverse genetics approach and the subsequent phenotype-driven screen to isolate hair graying animals, MSCs provides us an excellent opportunity for discovery of the genes required for stem cell maintenance.

8.4 Ontogeny of Melanocyte Stem Cells

During development, melanoblasts, precursors for pigmented melanocytes, emerge in the neural crest at around embryonic day 8.5 (E8.5). Melanoblasts migrate along the dorsolateral pathway between the dermatome and the overlying ectoderm, and from E10.5 migrate ventrally through the developing dermis. At E14.5, melanoblasts begin to invade the overlying epidermis and then migrate into the newly developing hair follicles (Quevedo and Holstein, 1992). Once melanoblasts enter into the follicles, they are segregated into two populations: one is mature melanocytes that localize at the hair matrix whereby they differentiate into mature pigment cells; the other is MSCs that reside at the LPP of the hair follicle and stay immature status until they are stimulated to undergo differentiation. As described in the previous section, *Bcl2*-/- mice are born normal pigmentation without having MSCs in the LPP of the hair follicle (Nishimura et al., 2005). This clearly indicate the first wave of melanogenesis initiates directly from the melanoblasts entering into the hair follicle without passing through MSCs, whereas melanogenesis in the subsequent hair cycles is derived from MSCs. The fact that MSCs are generated after the initiation of the first melanogenesis reminds us of an idea that the emergence of MSCs is independent of melanogenesis, and requires developmentally distinct molecular events that are diverged from melanocyte differentiation pathway (Mak et al., 2006). This concept that stem cell emergence is occurred after tissue development is likely to be common among many stem cell systems. It has been recently shown that, during testicular development, the first round of spermatogenesis is directly derived from gonocytes (precursors for spermatogonia cells) rather than from undifferentiatated spermatogonia, while subsequent cycles of spermatogenesis is derived from Ngn3-positive spermatogonial stem cells (Yoshida et al., 2006). In muscular development, Pax3/Pax7-double positive myogenic precursors give rise to skeletal muscle prior to the appearance of satellite cells at later stage of embryonic development (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). Precursors for hematopoietic cells are first appear in embryonic yolk sac, while definitive hematopoietic stem cells are generated at the much later stage of embryogenesis (Samokhvalov et al., 2007). Like a chicken-versus-egg argument, the question of which come first, the stem cells or the niche, is the matter of critical debate. The fact that the stem cell comes later during tissue development may support the idea that the differentiated progenies, acting as forerunners, create the niche first and then the tissue precursors colonized at the niche become stem cells.

When and how stem cells are generated during tissue development are one of the central questions for stem cells biology. Availability of Dct-LacZ transgenic mice, in which individual melanoblasts are marked by the specific expression of a LacZ reporter driven by melanocyte-specific Dct promoter (Jordan and Jackson, 2000; Mackenzie et al., 1997), allow us to address these questions by observing the process of the emergence of MSCs during hair follicle development. MSCs are defined as the resting melanoblasts that are resistant to an antagonistic antibody against c-Kit (Ack2) (Nishikawa et al., 1991). It has been shown that the

survival of proliferating melanoblasts is strictly dependent on the signaling from c-Kit tyrosine kinase receptor (Reid et al., 1995). In fact, treatment of newborn mice with Ack2 depletes proliferating melanoblasts/melanocytes from the epidermis, whereas, in the guard hairs that develop earlier than other hair follicles, several Ack2-resistant melanoblasts are detectable at the LPP of the hair follicles (Nishimura et al., 2002). These c-Kit independent melanoblasts are MSCs because hair pigmentation in the next hair cycling is detectable only in the guard hairs in which the melanoblasts are remained at the LPP after the Ack2 treatment, while hairs formed from the late-developing hair follicles are largely unpigmented (Nishimura et al., 2002). From these observations, it is shown that the earliest MSCs are established in the guard hair follicles by the end of embryogenesis (Nishimura et al., 2002). In parallel with these observations, whole mount staining of the Dct-LacZ hair follicles shows that melanoblasts are colonized at the LPP of the guard hairs from E18.5 onset (Fig. 8.3). In addition, it has been reported that, in Bcl2-/- epidermis, loss of melanoblasts at the LPP becomes evident in the guard hair during E18.5-P2 (Mak et al., 2006). Hence, taken together, these data suggest that the earliest MSCs are generated during E18.5-P2 at the LPP of the guard hair follicles.

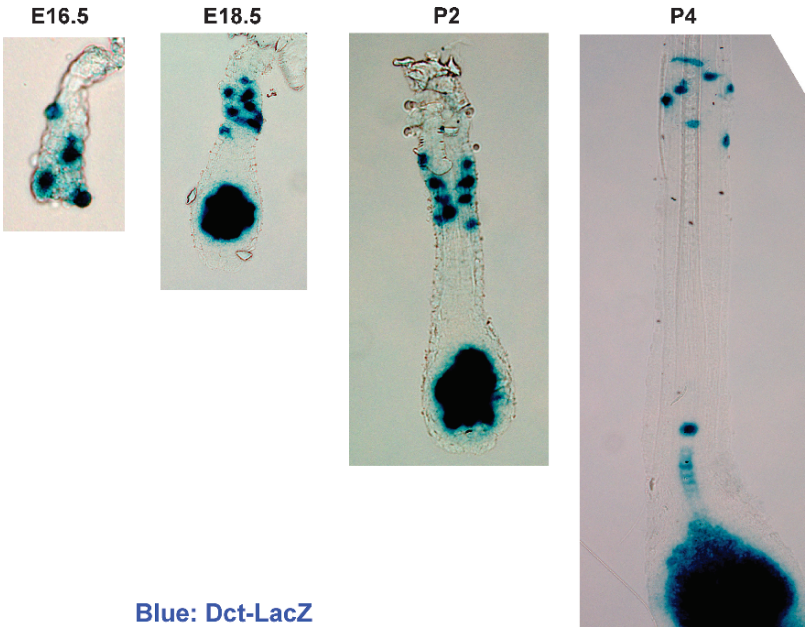

Fig. 8.3 Melanoblasts are colonized at the lower permanent portion from the E18.5 onset. A single guard hair follicle was isolated from each developmental stage of Dct-LacZ skin and stained with LacZ staining. Colonization of melanoblasts at the lower permanent portion was observed from the E18.5 onset

The next obvious question is how MSC fate is established during hair follicle development. Given the fact that embryonic melanoblasts give rise to both differentiated melanocytes and MSCs, there may be two alternative possibilities available in the emergence of MSCs: one possibility is that melanoblasts are heterogeneous in terms of their capacity to be MSCs and a particular pre-determined subset of melanoblasts is committed to be a MSC fate; the other possibility is that embryonic melanoblasts are homogenous population in which individual melanoblasts are bi-potential and the only melanoblasts that colonize at the LPP are induced to become MSCs by the external signaling. The capability of reconstituting MSC population by the clonally cultured melanoblasts may support the latter possibility (Yonetani et al., 2007). Consistent with this notion, it has been shown previously that the niche plays a dominant role in the fate determination of MSCs (Nishimura et al., 2002). Therefore, it is most likely that melanoblasts are intrinsically bipotential and the local signaling from the LPP may play an important role in determination of MSC fate.

What is the role of the LPP in fate determination of MSCs? One potential answer for this question may be obtained from our studies on Bcl2-/- mice. Bcl2-/- mice exhibit no obvious defect in the hair pigmentation in the first hair cycle, while showing premature hair graying from the second hair cycle. This phenotype of Bcl2-/- mice indicates that Bcl2 is selectively essential for the MSCs, whereas its function for the embryonic melanoblasts and mature melanocytes is dispensable. It has been demonstrated that Bcl2 plays a critical role in the protection of cells against growth factor deprivation-induced apoptosis. SCF is a specific ligand for c-Kit receptor, and plays an essential role for the survival of both melanoblast and melanocytes. As it has been shown that SCF expression in the basal epidermis is down-regulated after the birth (Yoshida et al., 1996), we reasoned that loss of MSCs in Bcl2-/- mice is due to incomplete protection of MSCs when SCF is downregulated in the LPP. To demonstrate this idea, we crossed Bcl2-/- mice with K14-SCF transgenic mice (Kunisada et al., 1998), in which SCF is constitutively expressed in the basal keratinocytes. In supporting this idea, we demonstrated that loss of MSCs in Bcl2-/- hair follicles are rescued in Bcl2-/-; K14-SCF mice (Mak et al., 2006), indicating that Bcl2 plays an indispensable role in the protection of MSCs in the absence of SCF/c-Kit signaling. In consistent with these data, requirement of both Bcl2 and c-Kit signaling has been also shown in hematopoietic stem cells (Domen et al., 2000). As the resting MSCs are independent on SCF/c-Kit signaling, our data suggest that Bcl2 is required temporary by the time before MSCs enter a resting status (Mak et al., 2006). In this context, it is noteworthy that the premature hair graying phenotype of Bcl2-/- mice is rescued in the combinatory knockout mice of both Bcl2 and Bim genes (Bcl2-/-; Bim-/-) (Bouillet et al., 2001). As Bim is a pro-apoptotic factor whose expression is upregulated in response to growth factor deprivation (Biswas et al., 2007), this data also support our idea that Bcl2 is transiently required for the survival of MSCs to protect them from growth factor deprivation-induced apoptosis when SCF expression is downregulated in the LPP during hair follicle development.

8.5 Molecular Characterization of Melanocyte Stem Cells at the Niche

Understanding the molecular mechanisms underlying the regulation of stem cells within the niche is critical for stem cell biology; however, this has been largely hampered by the lack of systematic approaches to dissect the complete molecular make-up of stem cells at the niche. Taking advantage of MSC system where individual stem cells are identifiable by the location, we isolated single MSCs from the hair follicles and determined gene expression signature of individual MSCs (Osawa et al., 2005). By crossing *Dct-Cre* knock-in mice (Guyonneau et al., 2004) with *CAG-CAT-EGFP* reporter mice in which a robust GFP expression is achieved under the control of a strong ubiquitous promoter, *CAG*, after Cre-mediated removal of a *chloramphenicol acetyl transferase* (*CAT*) gene cassette (Kawamoto et al., 2000), we engineered transgenic mice to express GFP in a melanocyte-specific manner. These transgenic mice enable us to identify the individual MSCs by the GFP expression and their location. We picked single MSCs from the LPP of the microdissected guard hairs and performed gene expression profiling analysis using single-cell based whole transcript amplification technology. Although there is a technical limitation in unbiased amplification of the whole transcript, this could be the most accurate way to isolate stem cells directly from the niche.

The results indicate the downregulation of various key melanogenic genes including *Sox10, Mitf, c-Kit, Lef1*, and *Ednrb* is evident in MSC population (Osawa et al., 2005). As it has been well demonstrated that these genes play crucial roles in the proliferation, survival, and differentiation of both melanoblasts and melanocytes, these data suggest that MSCs utilize a different biochemistry from that of melanoblasts and melanocytes to maintain their physiological features. Interestingly, our global gene expression analysis also shows downregulation of several house keeping genes, suggesting an idea that basal transcription itself is globally suppressed in MSC population. Given the fact that majority of MSCs are kept in a quiescent state at the LPP, such global downregulation of transcription in MSCs is supposed to be reflecting one aspect of cellular quiescence as it has been characterized that cellular quiescence is a state accompanied by lower rates of transcription, translation, and metabolism (Yusuf and Fruman, 2003). Consistent with this notion, similar global repression of transcription has been reported in yeast at the stationary phase, a quiescent state that is induced by the nutrient deprivation, due to shutdown of general transcription machinery (Radonjic et al., 2005), although its physiological role remains unclear.

In melanocyte lineage, it is known that both Sox10 and Mitf act as master regulatory transcription factors and play crucial roles in the induction of proliferation and differentiation of melanoblasts (Goding, 2000; Wegner, 2005), and, hence, it is obvious that downregulation of these molecules in MSCs causes negative impact on their proliferation and differentiation. Indeed, it is recently reported that down-modulation of *Mitf* level by a specific RNAi in a melanoma cell line results in cell-cycle

exit associated with stem cell-like smaller morphology (Carreira et al., 2006). Although the underlying molecular mechanism in the downregulation of *Sox10* and *Mitf* in MSCs are not clear, one potential explanation for their downregulation may be due to the inhibition of Wnt signaling at the LPP of the hair follicle. In fact, there are increasing number of evidences that demonstrate the link between Wnt signaling and *Sox10* and *Mitf* expressions. In *Xenopus* embryo, ectopic activation of Wnt signaling is shown to induce the upregulation of *Sox10* expression (Aoki et al., 2003). A crucial role of Wnt signaling in the regulation of *Mitf* has been also demonstrated in melanoblasts (Lang et al., 2005). Thus, by integrating the recent finding that several Wnt inhibitors such as *Dkk3, Sfrp1*, and *Dab2* are highly expressed in the LPP (Morris et al., 2004; Tumbar et al., 2004), it is speculated that downregulation of Wnt signaling in the LPP may represent the main cause of the downregulation of *Sox10* and *Mitf* in MSCs at the niche.

8.6 Role of Notch Signaling in the Maintenance of Melanocyte Stem Cells

Notch compromises a family of highly conserved receptors, whose activation is induced by their specific ligands, Delta and Jagged, through cell-cell interactions. Once activated, the intracellular domain of the Notch receptor (NICD) is cleaved by γ-secretase, resulting in translocation of the NICD into the nucleus. Subsequently, NICD is associated with the transcription factor RBP-J to generate the transactivation complex, which finally initiates transcription of target genes such as the hairy/enhancer of split (Hes) transcriptional repressors (Androutsellis-Theotokis et al., 2006). Accumulation of evidence indicates the pleiotropic roles of Notch signaling in various aspects of cellular regulation including cell-fate determination, cell cycle regulation, apoptosis, and stem cell maintenance. We found in our gene expression analysis that Notch signaling and its immediate target Hes1 are activated in immature melanoblasts including MSCs (Moriyama et al., 2006). To clarify the role of Notch signaling in the regulation of MSCs, we conditionally ablated *RBP-J* gene in a melanocyte-specific manner (*RBP-J* cKO). *RBP-J* cKO mice showed severe coat color dilution in the initial hairs followed by loss of hair pigmentation after the first hair molting (Fig. 8.4). Detailed analysis of the hairs of *RBP-J* cKO mice revealed that the first hairs developed from the mice were a mixture of pigmented and unpigmented, whereas the hairs formed in subsequent hair cycle were completely unpigmented (Moriyama et al., 2006). Histologically, *RBP-J* cKO skin showed a dramatic reduction of melanocytes in the hair matrix and the virtually complete absence of MSCs in the LPP of the hair follicle. Thus, these data indicate that Notch signaling is essential for the maintenance of both melanoblasts and MSCs. In addition, histological analysis showed that melanoblasts undergo apoptosis in the absence of Notch signaling. Therefore, Notch signaling plays a role in promoting melanoblast survival by protecting from apoptotic cell death. To elucidate role of Hes1 at the downstream of Notch signaling, we generated transgenic mice in which *Hes1* is

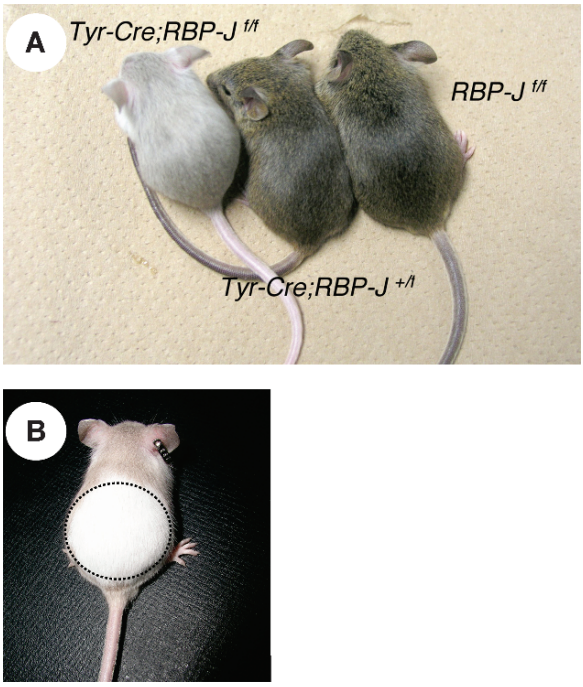

Fig. 8.4 Notch signaling is essential for the maintenance of melanocyte stem cells. In order to ablate Notch signaling in a melanocyte-lineage manner, we crossed Tyr-Cre transgenic mice with RPB-J flox/flox mice to obtain Tyr-Cre; RPB-J flox/flox mice. A Tyr-Cre; RPB-J flox/flox mouse showed severe coat color dilution in the hairs formed from the first hair cycle (A), and hairs formed in the second hair cycle were completely unpigmented (B). These data indicate that Notch signaling is essential for the maintenance of both melanoblasts and melanocyte stem cells

constitutively expressed in a melanocyte-lineage. Since loss of melanoblasts in the absence of Notch signaling is rescued in this transgenic skin, it is clear that Hes1 plays an indispensable role in the protection of melanoblast survival at the downstream of Notch signaling. Given the fact that Hes1 is a transcriptional repressor, Hes1 may play a role in repressing gene expression program required for the initiation of apoptotic cell death (Moriyama et al., 2006).

Consistent with our data, it has been recently shown that mice in which both *Notch1* and *Notch2* are sequentially disrupted in melanocyte-specific manner exhibit age-associated premature hair graying (Schouwey et al., 2007), indicating a dose-dependent fashion of the requirement of Notch signaling in the maintenance of MSCs. This result suggests a possibility that the attenuation of Notch signaling may represent one of the causes of age-associated progressive hair graying, as it has been demonstrated that diminished activation of Notch signaling in the aged muscle satellite cells (=stem cells for muscle) causes reduction of their regenerative capacity of injured muscle in aging mice (Conboy et al., 2003).

It is widely accepted that stem-cell aging and its associated stem-cell dysfunction contribute to altered regeneration capacity of aged tissue. Recent studies on stem-cell aging have provided compelling evidences that elevation of reactive oxygen species (ROS) accelerates its aging, and anti-oxidative defense in stem cells plays a key role to maintain their longevity and functions (Ito et al., 2006; Tothova et al., 2007). Although the scientific proof has not been established yet, it has been widely recognized that various environmental and endogenous stresses such as chronic physical and psycho-emotional stress accelerate the onset of human hair graying (Arck et al., 2006). Since all these stresses ultimately induce physiologic oxidative stress at their downstream, it is likely that there are some correlations between oxidative stress and hair graying. Given a link between attenuation of Notch signaling and premature hair graying, one possible role of Notch signaling may be to protect MSCs from stress responses, although the exact molecular mechanism underlying the maintenance of MSCs by Notch signaling has been still unclear.

8.7 Melanocyte Stem Cells: As a Model to Study the Relative Quiescence of Stem Cells

Numerous evidences indicate that MSCs at the LPP are kept in a quiescent state. They are infrequently dividing and in a c-Kit-independent resting state. It is also shown that the expressions of various molecules required for melanocyte activation are maintained at low level. How come these properties of MSCs are maintained?

In a steady state tissue in adults, the majority of stem cells in the niche are thought to be kept in a quiescent state, while these stem cells are capable of undergoing intensive proliferation when stimulated (Suda et al., 2005). Cellular quiescence is a state characterized by decreased cell-cycle progression and metabolic activity. There is growing evidence that the relative quiescence plays a crucial role in protecting stem cells from accumulating metabolic damage as well as genotoxic stress to ensure their long-term survival (Kobielak et al., 2007; Rossi et al., 2007; Tothova et al., 2007). For instance, recent loss-of-function studies demonstrate that the cell-cycle progression of stem cells is negatively regulated by several cell cycle inhibitors, such as p21, p27, and Pten (Cheng et al., 2000; Groszer et al., 2001; Walkley et al., 2005). Indeed, the loss-of-function of p21 in mice leads to the dramatic acceleration of proliferation of hematopoietic and neural stem cells, resulting in exhaustion of the stem cells (Cheng et al., 2000; Kippin et al., 2005). Thus, the cell cycle withdrawal by the cell-cycle inhibitors plays a key role for the maintenance of stem cells pool by protecting from their mitotic stress-induced exhaustion. In addition to these cell-cycle inhibitors, the quiescent status of stem cells is also maintained under the control of active transcriptional regulation. One example for such transcription factors is a family of transcription factors FoxO, a member of Forkhead or winged-helix DNA binding (Fox) transcription factor, class O. FoxOs, which were originally identified for the responsible molecules found in the chromosomal translocation breakpoints associated with tumors, plays an important

role in diverse physiologic processes, including induction of cell cycle arrest, apoptosis, and stress resistance (van der Horst and Burgering, 2007). Conditional ablation of FoxOs causes loss of hematopoietic stem cells due to the acceleration of cell-cycle progression and the induction of oxidative stress-induced apoptosis (Tothova et al., 2007). Hence, it has been demonstrated that FoxOs transcription factors play an essential role for the protection of quiescent stem cells from the cell-cycle progression and metabolic stress. These studies provide strong evidences for the essential roles of stem-cell intrinsic "quiescence factors" in the maintenance of quiescent state of stem cells. Without having such "quiescence factors", the relative quiescence of stem cells is lost: stem cells are being constitutively activated; ultimately exhausted by metabolic damages such as oxidative stress (Tothova et al., 2007). However, given an irresistible role of the niche in the maintenance of stem cells, these intrinsic genetic programs must be subject to the endogenous regulations to ensure appropriate control of stem cells by the niche. Despite the recent remarkable progress in understanding roles of stem cell-intrinsic factors, the extrinsic mechanisms involved in the induction and/or maintenance of the quiescent state of stem cells remain poorly understood. Therefore, one important direction for future stem cell research could be to clarify environmental signals that induce the quiescent state of stem cells.

In this regard, it could be intriguing to address the question why the relative quiescence of stem cells is conserved across both animal and plant kingdoms (Benfey, 1999). In lower organisms such as *Yeast* and *C. elegans* (Fitch, 2005; Gray et al., 2004), it is well known that cellular quiescence is induced by adverse conditions such as nutrient starvation. Intriguingly, in these organisms, the cellular quiescence also serves as a critical program to gain cellular longevity against aging. Hence, it is likely that the cellular quiescence has been evolved by means of elongation of cellular longevity against adverse condition. Recent studies on cellular aging have revealed that the core program to protect against cellular aging is probably conserved throughout unicellular organisms to mammalians and may be also important for maintenance of stem cell longevity (Guarente and Kenyon, 2000). It has been well accepted that ROS generated through mitochondria metabolism represent a main cause of cellular aging, and the only way to escape from ROS-induced aging may force the cells in a quiescent state to reduce metabolic damage (Guarente and Kenyon, 2000). From the analysis of MSCs, we propose that the niche is an environment that insulates stem cells from various activating stimuli, and maintains quiescent state of stem cells to ensure their long-term survival.

In this review, we featured on the suppressive role of the niche in the regulation of MSCs. It is obvious from numerous evidences that the niche acts dominantly to determine stem cell and maintain their quiescent state (Nishimura et al., 2002). However, the niche itself could be more flexible, and its dominant suppressive role may represent just only one aspect of the whole picture of the stem cell niche, otherwise stem cell never get activated. Because stem cells are capable of undergoing rapid proliferation upon stimulatory signaling particularly when they regenerate and replenish a tissue after tissue damage, it could be possible that the function of niche is switchable from the growth suppressive niche to growth stimulatory one.

As we describe in this review, MSCs have various advantages to clarify molecular mechanism underlying the maintenance of stem cells. There are several drawbacks in current stem cell research. By employing MSCs as a model, one can expect to overcome such drawbacks in studying stem cell biology. Therefore, MSCs provide us one of the best models for studying the complex interplay between environmental cues and intrinsic factors that underpin stem cell fate.

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Chapter 9
In Vitro hESC Technology: State of the Art and Future Perspectives

William Lathrop Rust

Abstract The remarkable capacity of human embryonic stem cells (hESC) to transform into functional human tissues has captured the imagination of biologists and non-biologists alike. Unfortunately, the mechanisms of this transformation are still poorly understood and current methodology is inadequate to direct the formation of many medically useful and safe cell therapies. This chapter provides an analysis of the state of the art of *in vitro* hESC research aimed at generating tissues suitable for the clinic. Discussed are laboratory techniques for controlling differentiation, for purifying homogenous populations, and for editing the genome of hESC. Also discussed are the strategies for ensuring the safety of hESC derived tissues for human patients, and an outlook on the future directions of hESC research.

Keywords Human embryonic stem cell, differentiation\, enrichment, embryologic development, transfection, transduction, homologous recombination, mixed chimerism, teratoma, nuclear transfer

9.1 Introduction

Human embryonic stem cells (hESC) are cell lines derived from outgrowths of the inner cell mass of five to seven day old human blastocysts, although derivation of hESC lines has been reported from as early as the eight cell embryo and as late as the morula stage embryo (Strulovici et al., 2007). The hallmarks of these cells are their remarkable capacity to grow in a state of developmental stasis and, with the appropriate stimulus, mature into any tissue of the adult body. In the undifferentiated state they express telomerase and are essentially immortal (Maitra et al., 2005; Hiyama and Hiyama, 2007). As differentiation proceeds, the lineage potential of

Institute of Medical Biology, Singapore Stem Cell Consortium, 11 Biopolis Way, #05-06 Helios, Singapore 138667
E-mail: William.rust@imb.a-star.edu.sg

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individual cells within the hESC culture gradually restricts according to biochemical and architectural guidelines that, presumably, recapitulates aspects of human embryologic development.

Investigators in the field of hESC research however, might adopt a more pragmatic view of these cells. That is, hESC are a precocious cell population with a natural tendency to differentiate uncontrollably in culture. The long term maintenance of pluripotent hESC in culture leads to genetic drift and the accumulation of chromosomal abnormalities (Draper et al., 2004a, 2004b; Hoffman and Carpenter, 2005). Indeed, the hESC researcher must take care to keep a high quality culture of undifferentiated, karyotypically normal hESC cells and a great deal of research is focused on developing protocols and reagents for this purpose. Additionally, all hESC lines are not equal. Investigators tend to have a favorite cell line for each purpose, as all lines do not respond equally to a given differentiation protocol (D'Amour et al., 2006; Phillips et al., 2007). Cross comparison between cell lines, and between varying passage numbers of the same cell lines, suggest that many discrepancies between cell lines may arise from heritable changes in the epigenome, such as DNA methylation and histone modification (Bibikova et al., 2006; Allegrucci and Young, 2007; Mikkelsen et al., 2007). This suggests that the epigenome of hESC is relatively unstable.

The origins of modern hESC research can be traced to observations made in the 1950's of mouse testicular teratomas, which had the peculiar ability to form multiple cell types of the adult body (reviewed in Solter, 2006). The pluripotent and embryonic nature of cell lines isolated from mouse teratomas and teratocarcinomas was ultimately proven by injection of these cells into the mouse blastocyst, which were implanted into pseudopregnant mice. The injected cells contributed, in some cases, to all tissues of the adult animal, including the germ line. These successes were followed in 1981 by the derivation of the first stem cell line directly from the mouse embryo. These cultures formed as outgrowths of the mouse blastocyst when plated on mitotically inactive stromal layer of fibroblast cells. Largely because they could be modified genetically *in vitro* and were highly efficient at producing chimeras when injected into mouse blastocysts, the embryonic stem cell became an excellent tool for the analysis of mammalian development. Not until isolation and culture of the first human embryonic stem cells in 1994 and 1998, respectively, did the principle research goal become the development of human cell therapy (Bongso et al., 1994; Thomson et al., 1998). Most techniques and reagents used in modern hESC research has benefited from these years of research in teratoma, embryonic carcinoma, embryonic germ cells, and murine embryonic stem cells. Many strategies for directing the differentiation of hESC are directly adapted from murine embryonic stem cell protocols. As this chapter is focused on hESCs, size constraints do not always allow appropriate homage to these essential research counterparts.

Most human ESC cultures are supported by a stroma of murine or human embryonic fibroblasts and require basic fibroblast growth factor (bFGF) to maintain the pluripotent state (Hovatta et al., 2003; Richards et al., 2003; Yoo et al., 2005). In order to reduce exposure to undefined reagents and xenogenic co-cultures,

feeder-free culture methods have been proposed. These include plating the cells on Matrigel matrix or fibronectin (Xu et al., 2001; Amit et al., 2004; Li et al., 2005). All of the influences from the feeder cells that maintain the undifferentiated state, however, are not yet identified, and, at the time of this writing, long-term feeder-free cultures still require that the medium be conditioned by fibroblast cells (Rajala et al., 2007).

The differentiation of the hESC culture *in vitro* is launched in one of three ways. Most commonly, the hESC colonies are manually or enzymatically disrupted to cell clusters containing many hundreds of cells which are transferred to suspension culture using low-adhesion plastic, spinner flasks, or hanging drops (Conley et al., 2004; Cameron et al., 2006). Within hours, these cell clusters form multi-layered structures displaying loose structural homology to the early mouse embryo, and are termed embryoid bodies (EBs) (Itskovitz-Eldor et al., 2000; Conley et al., 2004, 2007). Alternatively, undifferentiated hESC cultures are simply deprived of bFGF or are re-plated on an attachment substrate in the absence of bFGF. Using all three of these methods, the augmentation or complete removal of fetal calf serum is also employed to drive differentiation.

Once the culture restraints guarding the pluripotent state have been removed, the hESCs will spontaneously differentiate into a heterogeneous population of cells of varying lineages and developmental stage. Several uses of such heterogeneous populations of hESC are proposed. Assuming that spontaneous differentiation of hESC populations follows guidelines of ontogeny, these populations may be useful for modeling development of human tissues. As such, they are excellent sources for uncovering novel genes, proteins, or mechanisms involved in cell lineage specification (Laslett et al., 2007). Spontaneously differentiating populations may also be useful for predicting the toxicity or teratogenicity of compounds in pharmaceutical development, or identifying drugs which induce tissue regeneration (Cezar, 2007). Cell lines which contain a genetic mutation could be useful for modeling development of human genetic disease.

The ultimate goal of most hESC research, however, is to create human tissues which can be used to functionally replace damaged or diseased tissue of human patients. The supply of transplantable tissues from human donors is notoriously inadequate, and it is anticipated by some that hESC derived tissues will be capable of filling this need. For this application, pure populations of defined and characterized cell types are required. hESCs, therefore, are useful insofar as their differentiation can be controlled, or the desired cell types can be enriched to near purity. This chapter focuses on the state of the art of methods used to promote the differentiation of medically useful cell types within a population of differentiating hESCs, and the methods of enriching specific cell types from heterogeneous populations. The word “promote” is used loosely in this context as the direct impact of a particular agent on the differentiation of a cell type is not proven experimentally in most cases. It is possible that the promoting agent works indirectly, either by nurturing the development of other cells which have influence over the desired cell type, or by altering the non-cellular environment to create a niche that is permissive for differentiation of the desired cell type. In this case, the hESC are allowed to run

their course of spontaneous differentiation within limited confines. The major approaches to developing differentiation protocols are organized in this chapter into the following categories: Following embryologic cues; Co-culture; Niche-modeling; Empirical method; Genetic modification; and Enrichment. The target cell types referenced as examples of end-point differentiation are chosen by a pressing medical need, such as insulin-producing beta cells, cardiomyocytes, motor and dopaminergic neurons, and hematopoietic cells. As for any allogeneic transplant strategy, engraftment of hESC derived tissues must solve the challenge of immune rejection and must not pose a safety risk to the patient. A brief discussion of immune tolerance and safety strategies is also included.

9.2 *In Vitro* Differentiation: Following Embryologic Cues

It is rational that a sure method of deriving a mature cell from an embryonic one is to push it down the same developmental pathway that the cell would follow in utero. Indeed, gene expression analysis of spontaneously differentiating hESCs suggests that gene activation recapitulates, at least in sequence, that which is known of the developing mouse and human embryo (Zambidis et al., 2005; Beqqali et al., 2006; Rust et al., 2006)).

There are some logical arguments, however, to the contrary. Foremost is that the current understanding of human, or even mammalian, development is imprecise. The researcher therefore only has a very simplified view of the mechanisms of developmental biology at his disposal. It is even counter-intuitive that stem cells are being used to unravel the mechanisms of human development at the same time as this knowledge is being appropriated to control stem cell differentiation. Additionally, it is not known for certain to what extent hESC differentiation recapitulates ontogeny and which phenomenon are artifacts of *in vitro* culture. If *in vitro* and *in vivo* processes of differentiation were similar, however, some aspects of embryological development are impossible or impractical to replicate in the laboratory. For example, it is unlikely that an *in vitro* vasculature will be produced as a support system for the production of a transplantable whole organ (Strulovici et al., 2007). With these caveats in mind, however, the use of developmental cues learned from the study of mouse and other model organisms has proven highly successful as aids to controlling hESC differentiation *in vitro*, particularly for the derivation of the early embryonic germ layers.

The source and closest *in vivo* equivalent of the hESC is the inner cell mass of the blastocyst embryo. The inner cell mass, termed epiblast, gives rise to the three definitive germ layers of the embryo and some extra-embryonic tissues through the processes of cavitation and gastrulation. The application of factors which govern these processes *in vivo* are powerful tools for guiding the fate of hESCs *in vitro*.

In the developing mouse embryo, gastrulation commences with the formation of a transient groove on the future posterior of the epiblast termed the primitive streak (PS) (reviewed in (Gadue et al., 2005)). Juxtaposed epiblast cells emerge from the

streak and migrate in medial and anterior directions while concurrently acquiring endodermal (gastrointestinal tract, respiratory tract, liver, pancreas) and mesodermal (muscle, bone, connective tissue, circulatory system) fates. Definitive endoderm is formed from the anterior PS at the early and mid-PS stage, while mesoderm emerges from the mid and posterior PS. Epiblast cells which do not transit through the primitive streak become ectoderm (skin, neurectoderm) cells.

This cellular choreography is governed by gradients of soluble factors secreted from the extra embryonic tissue and the epiblast, which impose a spatial organization and hence provide a positional identity to the cells. One such important factor is BMP4, which is a TGFbeta family member produced by the proximal extra-embryonic ectoderm and is required for formation of the primitive streak. On hESC cultures, BMP4 is a key component of protocols which stimulates formation of the extra-embryonic trophectoderm, and the primitive streak-derived endoderm and mesoderm (Xu et al., 2002b; D'Amour et al., 2005; Phillips et al., 2007).

BMP4 signaling in the mouse embryo is antagonized by the inhibitors noggin, chordin, and follistatin, which are a product of the node and organizer regions, diametrically opposed to the site of BMP4 production (Munoz-Sanjuan and Brivanlou, 2002). Blockage of BMP4 signaling is sufficient in mouse and xenopus models for inhibition of the mesoderm and promotion of the neurectoderm. Likewise, application of noggin to hESC cultures increases the production of neural precursor cells and neurons, and decreases the occurrence of trophectoderm (Xu et al., 2002b; D'Amour et al., 2005; Sonntag et al., 2007).

A second key regulator of gastrulation is nodal, another TGFbeta family member, secreted from the anterior region of the primitive streak. Nodal is also required for formation of the PS and impacts formation of the PS-derived endoderm and mesoderm (Conlon et al., 1994; Gadue et al., 2005). Studies with mESC have shown that activation of the nodal receptors by Activin A preferentially forms endoderm (derived from the anterior PS) at high concentrations and mesoderm (derived from the more posterior PS) at lower concentrations. hESC cultures exposed to Activin A sequentially express genes characteristic of the primitive streak, definitive endoderm, and mesodermal cells (Schuldiner et al., 2000; D'Amour et al., 2005; Phillips et al., 2007). The cultures containing definitive endoderm cells produced greater proportions of the endodermally derived cells of the pancreas (D'Amour et al., 2006; Phillips et al., 2007).

As a last example, the signaling molecule retinoic acid is active late in gastrulation, and is implicated in patterning the various tissues of the foregut endoderm such as the precursors of the liver and pancreas, and also of posterior neural structures (Desai et al., 2004). After formation of endoderm by exposure to Activin A, retinoic acid was applied to hESC cultures to encourage differentiation of pancreatic progenitors (Jiang et al., 2007b; Shim et al., 2007). Sonic hedgehog, a key embryonic regulator of neural patterning, encourages neural fates in hESC cultures (Lee et al., 2007; Soundararajan et al., 2007). Applied to cultures which had been encouraged towards neural induction by application of sonic hedgehog agonists, retinoic acid induced formation of motor neurons (Lee et al., 2007; Soundararajan et al., 2007).

9.3 *In Vitro* Differentiation: Co-culture

A strategy closely related to that of following embryologic cues is to provide the hESC cultures with a cellular influence akin to what may be encountered by the developing target tissue *in vivo*. The neighbor tissue may encourage the undifferentiated cells to adopt a biologically aligned cellular identity. In this case, the causative factors directing differentiation are unknown. These factors can be a combination of extracellular matrix, direct cell-cell contact, or modification of the medium.

The definitive endoderm and mesoderm originating from the primitive streak are juxtaposed along the anterior-posterior axis. Interactions between these and the visceral endoderm contribute to patterning of the gut tube, cranial, and cardiac tissues (Gadue et al., 2005). Explant studies of the mouse and chick model systems show that the endoderm layer of the developing gastrula instructs the juxtaposed mesoderm to commit to the cardiac lineage (Garcia-Martinez and Schoenwolf, 1993; Sugi and Lough, 1994). Mummery et al. exposed an embryonal carcinoma (EC) cell line to the endoderm-inducing agent retinoic acid, and analyzed how the resulting cell lines influenced the differentiation of undifferentiated EC cells. One cell line with molecular characteristics of the visceral endoderm induced EC cells to form spontaneous beating cellular clusters with characteristics of cardiomyocytes (Mummery et al., 1991). This cell line, termed END2, was later shown to efficiently induce cardiogenesis from mESC and hESC, producing beating clusters of cells with characteristics of fetal ventricular and atrial cardiomyocytes (Mummery et al., 2002).

Another highly useful co-culture system takes advantage of the properties of bone marrow stromal cells to direct the differentiation of ESC towards hematopoietic lineages. The bone marrow is a specialized microenvironment that, in the adult, houses the stem cell niche for both hematopoietic and mesenchymal stem cells. These cells are therefore naturally equipped with the ability to nurture the development of stem cells towards erythroid, myeloid, and lymphoid fates. Co-culture of hESC with the murine bone marrow stromal cell lines S17 and MS5 produced erythroid and myeloid cells (Kaufman et al., 2001; Vodyanik et al., 2005). Other cells with similar capacities for directing hESC differentiation towards hematopoietic cells have been isolated from human bone marrow and human fetal liver (Qiu et al., 2005; Wang et al., 2005). These stromal cells, however, induce a predominance of macrophage commitment and a lack of lymphoid cells, a phenomenon that was linked to expression of macrophage colony-stimulating factor (M-CSF) (Nakano, 1995). In an effort to derive lymphoid cells, bone marrow was isolated from a mouse deficient in the M-CSF gene. A cell line generated from this tissue, termed OP9, was capable of directing the differentiation of mESC and hESC to the B and T cell lineages in addition to erythro- and myocyte lineages (Nakano, 1995; Cho et al., 1999; Vodyanik et al., 2005; de Pooter and Zuniga-Pflucker, 2007).

While the examples listed here represent cases wherein co-culture with a mature cell type directs the differentiation of hESC towards a predictable range of phenotypes, co-culture techniques have not been identified for directing the differentiation of

every desired cell. Indeed, the influence of a single type of support cell might not be sufficient to direct the differentiation of most mature cells. It is well known that injection of hESC into an immune suppressed host animal does not typically form anatomically aligned tissue, but instead forms a teratoma.

9.4 *In Vitro* Differentiation: Niche Modeling

Growth factors, hormones, and cytokines do not hold a monopoly on guiding cellular fate. In addition to these well known compounds, a growing body of literature shows that the microenvironment, such as the extracellular matrix, a three dimensional support, and even parameters such as gas pressure and the force of movement can have an impact on guiding cell specification (Bauwens et al., 2005; Philp et al., 2005). Research into adult tissue stem cells, particularly the skin stem cells, illustrates the impact of the niche on cell fate (Blanpain and Fuchs, 2006).

Matrigel, which is an extracellular matrix similar to that secreted by the primitive endoderm, blocks the formation of a native visceral endoderm layer normally formed by differentiating EBs, and increases the occurrence of gastrulation-like events (Rust et al., 2006). hESC cultures immobilized in a hydrogel modified to contain the cell adhesion ligand RGD peptide and VEGF produced more endothelial and other vasculature related cells than non-embedded EBs (Ferreira et al., 2007). Gerecht-Nir et al. found that embedding EBs into simple porous alginate scaffolds induced the formation of cells expressing vasculature markers (Gerecht-Nir et al., 2004). It is likely that as protocols for directing the differentiation of hESCs are refined, greater attention will be placed on the micro-environmental niche.

9.5 *In Vitro* Differentiation: Empirical Method

In addition to the strategies listed above, an empirical approach to finding factors capable of guiding the differentiation of hESC cultures has provided some surprising reagents not normally encountered by the developing embryo. For example, hESC cultures exposed to the demethylating agent 5-azacytidine produced a greater proportion of cardiomyocytes than control cultures (Xu et al., 2002a; Yoon et al., 2006). As chromosomal modifications are understood to play a great part in gene expression control, it is possible that global de-methylation of hESC cultures may be permissive to the spontaneous differentiation of particular cell types. Although only shown in primate ESCs the histone deacetylase inhibitor trichostatin A may operate by a similar mechanism to encourage the differentiation of cardiomyocytes (Hosseinkhani et al., 2007). The action of trichostatin A encourages the acetylation of histones and also the transcription factor GATA4. Acetylated histones allow greater access of transcription factors to DNA and GATA4 is implicated in cardiomyogenesis.

Another histone deacetylase inhibitor, sodium butyrate, is reported to encourage the differentiation of hepatocytes from hESC (Rambhatla et al., 2003). The cultures exposed to sodium butyrate, however, showed considerable cell death, indicating that sodium butyrate may be applying selective pressure. As hepatocytes are cells specialized in the detoxification of exogenous and endogenous substances, a mildly toxic environment could favor the survival of the hepatocyte. The popular cryoprotectant dimethyl sulfoxide (DMSO) has also been reported to encourage the formation of hepatocytes from hESC cultures and may operate by a similar mechanism (Soto-Gutierrez et al., 2006).

9.6 Genetic Modification

The full potential of hESC in the laboratory and in the clinic may only be achieved when differentiation strategies are coupled with modern methods of gene editing. The following examples illustrate how gene editing in hESC has produced valuable new cell lines and avenues of research. Constitutive transgenic expression of the pancreas-specific transcription factor Pdx1 in differentiating hESC cultures increased the occurrence of pancreatic cell types (Lavon et al., 2006). Selection of target cells based on the expression of a selectable transgene has been used to isolate several types of homogenous cell populations (see below). To ensure the safety of transplanted hESC-derived tissue, a failsafe suicide gene has been incorporated which can be activated should the transplanted tissue become tumorigenic (Schuldiner et al., 2003; see below). Coupled with the anticipated ability to clone a hESC cell, it should also be possible using gene editing techniques to produce a genetically repaired autologous tissue for patients suffering from an inherited genetic disorder (Rideout et al., 2002). For research purposes, the manipulation of fate regulating genes may be as useful for understanding human development as this technique has been in the mouse model (Kubo et al., 2004). As a last example, the incorporation of disease-specific mutations into hESC may be useful for modeling human disease progression. Achieving these results require robust methods of generating and selecting stably modified hESC cells.

The typical methods of introducing transgenes (electroporation, cationic lipids, and other cationic vectors), are relatively inefficient in hESC, and random integration into the genome is rare (Eiges et al., 2001; Zwaka and Thomson, 2003; Yates and Daley, 2006). A comparison of chemical transfection methods reported that the best yield of stable transfectants in hESC is around one per 10E5 (Zwaka and Thomson, 2003). Compounding this difficulty is the fact that hESC cultures are in general difficult to clone from single cells (Sidhu and Tuch, 2006). Despite these caveats, a clonal hESC cell line was manually isolated from a culture which had been electroporated with a plasmid harboring GFP driven by the human beta actin promoter (Costa et al., 2005). This line, termed Envy, had incorporated a single copy of the GFP cassette at a locus on chromosome twelve which did not disrupt any known genes, was uniformly expressed throughout the culture, and was not

silenced upon differentiation. In addition, Envy retained the markers of stem cells and the ability to differentiate, indicating that, in principle, hESC lines are amenable to genetic manipulation. Recently, robust expression of multiple transgenes in hESC has been demonstrated by transfection of a single plasmid containing two genes separated by the foot and mouth disease virus 2A segment, which encourages high level multi-cistronic protein expression from a single mRNA in human cells (Hasegawa et al., 2007). In this case, stable, undifferentiated hESC lines were reported which simultaneously expressed high levels of yellow fluorescent protein, cyan fluorescent protein, and red fluorescent protein.

To overcome the inefficiency of transfection and the rarity of gene integration events, viral transduction is used. Adenoviral vectors have been used to produce transient expression but these viruses do not integrate into the genome (Yates and Daley 2006). Lentiviral vectors, however, efficiently infect and integrate into the genomes of dividing and non-dividing cells and can achieve transduction of 80% of the hESC population at high multiplicity of infection (Suter et al., 2006). A modified version of a lentiviral vector harboring a nuclear scaffold attachment region to increase transgene expression achieved transduction rates of 87% at a multiplicity of infection of 12 (Ma et al., 2003). The site of integration of the lentiviral vectors, however, cannot be controlled. Lentivirus transduced constructs can therefore not be aligned behind native promoters or enhancers, nor can knockouts or site directed mutants be produced. In addition, promiscuous genome integration increases the chances of insertional mutagenesis.

Theoretically, the ideal system would be site directed homologous recombination, which would allow higher control over the expression of the transgene, allow creation of site-specific mutations, and limit deleterious effects on local gene expression patterns. Zwaka et al. were able to delete the HPRT gene from the X-chromosome of hESC cells after delivery via electroporation of a construct containing flanking homologous arms (Zwaka and Thomson, 2003). This spontaneous recombination of gene sequences containing homologous arms, however, only occurred in a fraction of successfully transfected cells. Using very large homologous regions may increase the rate of spontaneous recombination. This has been shown in mESC cells by transfecting large transgenic sequences on bacterial artificial chromosomes (Tomishima et al., 2007).

More efficient site directed recombination in hESC has been demonstrated using Cre recombinase and phage lambda integrase (Tan and Droge, 2005). These systems, however, only integrate the transgene where either the loxP or the lambda integrase attachment sites have been previously delivered, and require coordinate expression of the recombinase enzymes. Once these cell lines are engineered, however, recombination of novel transgenes can be highly efficient. For example, the recombination of transgenes into hESC already containing the loxP sites has been reported to occur at nearly 100% efficiency when the cells were transfected with the target sequence and cell permeant version of an active Cre recombinase protein (Nolden et al., 2006). Another site-specific recombination technology that may be applicable to hESC cells is the use of zinc finger nucleases (Urnov et al., 2005). In this case, zinc-finger DNA binding proteins which are tailored to recognize a

specific DNA sequence are coupled to a non-specific nuclease. At the site of zinc finger binding, the nuclease cleaves a double strand break. The break is subsequently repaired via homology directed repair using a sequence from a co-transfected plasmid. A final method used for insertion through recombination in hESC cells is the so-called "Sleeping Beauty" transposon system wherein the transposon containing flanking inverted repeats is transfected with a transposase gene which effects insertion (Wilber et al., 2007). A potential drawback of this system is that the sites and frequency of integration is random.

An alternative to creating stable integration of the transgene is to transfect the hESC with an episomally propagated vector modeled on the chromosome of the Epstein Barr virus (EBV) (Ren et al., 2006). Retention and expression of the EBV plasmid requires the trans-acting element EBV nuclear antigen 1 (EBNA1). A hESC line with stable integration of EBNA1 into the genome and subsequently transfected with a transgene on an EBV plasmid produced more stably expressing cell lines than did transfection of a standard transgene plasmid alone. Another advantage of this system is that the EBV plasmid can harbor large transgene sequences and does not cause insertional mutagenesis.

9.7 Enrichment

The state of the art of directed differentiation strategies can at best produce populations of cells that have a higher incidence of the desired cell type than control populations. To derive homogenous cultures of individual cell types, a strategy for purification of the target cells is required. The presence of a surface expressed protein that is unique to the target cell type is the ideal anchor for isolating the cells by either fluorescent activated cell sorting (FACS) or magnet assisted cell sorting (MACS). Blood lineage cells are frequently defined by expression of cell adhesion molecules and cluster of differentiation (CD) molecules expressed on the plasma membrane and are the most readily purified from differentiated hESC cultures.

A population of cells expressing the adhesion molecules PECAM-1 (CD31), Flk-1, and VE-Cadherin in differentiating hESC cultures was shown to be a precursor of cells with hematopoietic potential (Wang et al., 2004). Isolation of these cells by FACS produced a population of which 98% activated the ubiquitous hematopoietic marker CD45 (Wang et al., 2005). From longer differentiated cultures, MACS isolation of cells based on expression of CD45, or the co-expressed hematopoietic stem cell marker CD34, produced cells with myeloid and erythroid potential to a purity of up to 95% (Kaufman et al., 2001; Vodyanik et al., 2005; Woll et al., 2005). Each of these populations was shown to be proliferative *in vitro* and produced colony forming units characteristic of hematopoietic precursor cells isolated from somatic sources. When used as an anchor to isolate cells under alternate differentiation conditions, PECAM-1 isolation by FACS produced a population of endothelial cells capable of forming vascular structures *in vitro* and *in vivo* (Levenberg et al., 2002). This particular cell type may represent the precursor cell

of both the vascular and the hematopoietic lineages, termed the hemangioblast (Kennedy et al., 2007).

Other native surface molecules which have been useful for the purification of cells from differentiating hESC colonies are CXCR4, and NCAM (CD56). CXCR4 is a chemokine receptor found on early endodermal and mesodermal tissue. When used to FACS isolate cells subjected to a protocol which induces definitive endoderm differentiation, nearly 100% of the isolated cells expressed endodermal markers (D'Amour et al., 2005). MACS or FACS sorting of NCAM, an adhesion molecule characteristic of developing and adult neurons, produced neural populations of high purity (Carpenter et al., 2001; Pruszak et al., 2007). In addition, these investigators identified the surface antigen A2B5 and CD24 as candidates for better isolating neural precursors.

In most cases, the identity of a unique surface marker amenable for the isolation of specific human cell types is unknown. There are a few examples, though, where it was possible to sort a cell type based on an easily identifiable morphology. Observing that NCAM expressing neural lineage cells emerged from identifiable clusters in the hESC culture, Reubinoff et al. were able to generate populations which were 83% neurons and glia via manual isolation and sub-culture (Reubinoff et al., 2001). An enrichment of cardiomyocytes to 70% was achieved by isolating a dense cell fraction produced by applying single cells of differentiated cell populations over a percoll gradient (Xu et al., 2002a; Xu et al., 2006).

Barring the presence of a native cellular characteristic which is amenable to cell sorting, cell populations may be purified by introducing a marker transgene which is only expressed in the target cells. For example, hESC cultures were stably transfected with eGFP driven by the albumin minimal promoter (Lavon et al., 2004). Upon differentiation, cells which had acquired a hepatocyte cell fate expressed albumin, activated eGFP expression, and were sorted by FACS to achieve a population purity of 95% mature and immature hepatocytes. A similar strategy was used to isolate cardiomyocytes by placing eGFP behind the control of the cardiac specific myosin light chain 2v promoter (Huber et al., 2007). Alternatively, a cell trap method may be used. Although not yet reported in hESC, this strategy has been successful at purifying cell subpopulations from mESC cultures. Klug et al. transfected mESC cells with neomycin resistance driven by the alpha-cardiac myosin heavy chain promoter (Klug et al., 1996). After differentiation towards the cardiac lineage, cultures were exposed to G418. Non-cardiac cells not expressing neomycin resistance perished, leaving a population composed of greater than 99% cardiomyocytes. To isolate neural progenitors from mESC, neomycin resistance was integrated by homologous recombination into the Sox2 transcription factor gene (Li et al., 1998). After neural induction, application of G418 produced a population, greater than 90% of which expressed neural cell markers. As an alternative to expression of GFP or neomycin resistance, it is also possible to drive expression of an innocuous cell surface molecule by a tissue specific promoter, and isolate those cells by FACS or MACS (Orchard et al., 2002).

As protocols for the derivation of specific cell types from hESC cultures are developed, it is likely that many more successful enrichment strategies for hESC

cultures will be reported. For these strategies to be most effective, however, it is important that the sorted population is proliferative *in vitro*, allowing for cell expansion. If a genetic strategy is adopted, it is also important that the transgene does not elicit an immune reaction after transplantation.

9.8 Safety and Anti-rejection Strategies

Any tissue or agent intended for injection into a human patient must meet rigorous safety guidelines as defined by the U.S. FDA, and other governmental agencies. hESC cells present a host of compounding safety challenges. One of which is assurance that the cells do not harbor any adventitious agents which may have been acquired through exposure to xenogenic or non-controlled reagents. To achieve this goal, the derivation of hESC cell lines using defined, non-xenogenic reagents and under conditions which meet the standards of the U.S. current good manufacturing practice (cGMP) guidelines has recently been announced (Crook et al., 2007). A second safety challenge is the demonstration that the transplanted tissue is not capable of forming a teratoma or a tumor, a natural characteristic of undifferentiated hESC (reviewed in Hentze et al., 2007). Using mouse ESCs, it was shown that as little as two pluripotent mESCs transplanted into the flank of nude mice are sufficient to initiate a tumor (Lawrenz et al., 2004). It is, however, widely established that the greater the differentiation, the lower the tumor-forming potential of the hESC culture (Brederlau et al., 2006). Following this logic, isolation of differentiated cells from hESC populations might go a long way towards eliminating the tumor risk. However, data from long-term transplantation with human hESC is lacking. It must also be noted that testing hESC ability to form a tumor when transplanted into a mouse or other immune suppressed non-human mammal may not be representative of the risk to a human patient.

One strategy proposed to act as a failsafe for the tumor inducing potential of hESC is the incorporation of a suicide gene that could be activated to ablate the transplanted tissue should a health risk arise (Schuldiner et al., 2003). Schuldiner et al. engineered a hESC line to constitutively express the herpes simplex virus thymidine kinase (HSV-tk) gene. Active HSV-tk in the transplanted cells phosphorylates the antiviral drug ganciclovir, converting it to a highly toxic form. As proof of principle, ganciclovir administered to SCID mice completely eliminated tumors formed by the transgenic hESC.

Another safety issue to be addressed is the capacity of the transplanted cells to stimulate an immune response in the patient. Therapeutically useful cells derived from hESC express the highly immunogenic major hisocompatibility I (MHCI) molecule, and other minor alloantigens, and would therefore be rejected from non-immune privileged sites in the human body (reviewed in (Drukker and Benvenisty, 2004)). As the derivation of patient specific hESC lines has not yet been reported, engraftment of hESC without general immune suppression will require a strategy to achieve immune tolerance. One method of achieving this is by creating a condition

termed mixed hematopoietic chimerism. This is a condition wherein hematopoietic repopulating stem cells from an allogeneic donor are transplanted into the bone marrow of myeloablated patients. The transplanted stem cells create leukocytes which co-exist with the host immune cells. If the patient were to receive a transplant from the same donor, the donor antigen presenting cells within the thymus would suppress host T cells which would recognize the transplant as foreign. Therefore, hESC-derived tissues could be tolerated by an allogeneic recipient if the patient also received hematopoietic stem cells derived from the same hESC line.

The formation of patient-specific tissues which could be accepted without extra intervention ultimately requires the creation of genetically identical stem cells, either from cloned human blastocysts or by the process of parthenogenesis. Mammalian clones are normally produced by the transfer of nuclei from somatic cells into enucleated unfertilized oocytes. The somatic cell genome is re-programmed by unidentified factors in the oocyte cytoplasm to be capable of growth into a blastocyst (reviewed in Meissner and Jaenisch, 2006). Aside from the fact that cloning by nuclear transfer has not been achieved with human tissues (at the time of this writing), the scarcity of human eggs for research hampers research efforts. Another technique, not using oocytes, has been used to create cloned stem cells from mice (Egli et al., 2007). With this technique, metaphase phase chromosomes of a somatic cell were transferred to an arrested metaphase stage zygote from which the chromosomes had been removed. Factors from the cytoplasm of the zygote re-programmed the transplanted somatic chromosomes such that a cloned stem cell line could be harvested. This work may pave the way for similar success of the first cloned human stem cell line. Although the mitochondrial genome contained by these cloned hESC would be genetically distinct, studies have suggested that engraftment into the host without immune rejection could still occur (Lanza et al., 2002). Lastly, it may also be possible to use stem cells derived from a parthenogenic blastocyst. Parthenogenesis is the phenomenon whereby an unfertilized oocyte is induced to divide to form a diploid pluripotent cell population. Tissues derived from these cells would be a perfect match to the donor but would only be available to female patients and would contain only homologous chromosomes, potentially unmasking deleterious recessive alleles (Drukker et al., 2006).

9.9 Discussion

As hinted to throughout this chapter, the state of the art of *in vitro* hESC technology is still relatively immature. As demonstration of this generalization, convincing data of hESC derived cells functionally repairing damaged tissues in animal models is rare. For example, several labs have reported generating glucose-responsive insulin secreting cells from hESC, but none have yet reported recovery of a diabetic mouse after transplantation (D'Amour et al., 2006; Santana et al., 2006; Jiang et al., 2007a, 2007b; Phillips et al., 2007). Several labs have also reported the engraftment and electrical coupling of hESC derived cardiomyocytes into rat hearts (Dai et al.,

2007; Laflamme et al., 2007; Tomescot et al., 2007; Xie et al., 2007). Those reporting functional recovery after transplantation of the hESC tissues into the damaged area of infarcted rat hearts, however, show only a limited amelioration of heart function, or formation of teratoma (Hodgson et al., 2004; Cai et al., 2007; Laflamme et al., 2007; Leor et al., 2007).

Another generalization which can be made concerning the state of the art is that although many tissue types have been identified in hESC cultures, they are frequently immature. This could reflect the fact that clusters of differentiating hESC cells create an environment that is more proximal to that of the early embryo than that of the adult. Several factors are lacking from the environment wherein a terminally differentiated tissue develops, such as three dimensional functional organs, circulatory and lymphatic systems, and a host of hormones and physical pressures. Because of the inability to create the perfect niche, the strategy of some researchers is to inject only partially differentiated cells *in vivo*, assuming that a committed cell will complete its differentiation once transplanted to the mature organ (Tomescot et al., 2007).

Another question at the heart of hESC research is the compatibility of hESC derived tissues for human transplantation. It must be remembered that hESC, as an immortal, developmentally static cell line cultured in two dimensional plastic dishes, is an artificial creation of the laboratory. Therefore, hESC differentiated cell types which display characteristics of adult cells may still harbor a host of unknown *in vitro* artifacts that will only become manifest at the time of transplant and incorporation into human patients. Regardless, it should also be clear that in the relatively short time frame since hESC lines have been cultured, great strides have been made in understanding their character and in controlling their differentiation. More importantly, greater human and financial resources are being devoted to hESC research. hESC cells still represent, today more than ever, a highly promising source of tissues for transplant into human patients. It must also be noted that transplantation of adult stem cell tissues such as bone-marrow derived mesenchymal stem cells is occurring today and success of these trials will help pave the way for acceptance of hESC derived tissues in the clinic (Yeager, 2002; Burt et al., 2003; Oyama et al., 2003).

9.10 Future Perspectives

Without making predictions of the timeline of the first successful human therapy by the transplant of hESC-derived tissues, it can be expected that the near future holds a series of exciting and ground-breaking hESC research successes. These successes could include the first successful creation of a hESC line from a cloned human blastocyst or the long-term and complete rescue of animals from spinal cord injury, heart trauma, or diabetes. It is also possible that the earliest medical impact from hESC technology will not come from hESC derived tissues but rather from hESC-derived reagents or from information garnered by studying hESC behavior

(Cezar, 2007). For example, understanding the machinery that gives the stem cells their character may inform how to induce the regenerative capacity of adult human tissues. Certainly, the evolution of this research and the rate of its progress will also be molded by the ongoing ethical debates surrounding the derivation, research, and use of hESC. For example, one subject of current debate is the use of hESC to form chimeric animals harboring human tissue. If injected into the blastocyst of a non-human mammal, hESC could, in theory, be able to contribute to adult tissues of the ensuing animal if it were allowed to develop to term. If the current state of hESC technology does not permit the formation of transplantable organs *in vitro*, it is hypothetically possible that a chimeric animal could be used to grow human organs for harvesting. In any case, the progress shown by investigators in the hESC field, and the potential medical impact of the data generated warrants continuing and vigorous research into hESC technology.

Although this chapter covers a broad range of topics related to hESC cells *in vitro*, there are still a number of related and exciting subjects not mentioned. Omitted subjects worth investigating include the derivation of hESC lines, the various techniques for the expansion of hESC cells, the epigenetic status of hESC cells, the creation of stem cell banks containing HLA-typed hESC lines, the creation of stem cells by parthenogenesis, the definition of the genes which are responsible for "stemness", and the ethical debate surrounding the derivation, research, and use of hESC lines.

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Chapter 10
Tumor Stem Cells: How to Define Them and How to Find Them?

Ibrahim Alkatout and Holger Kalthoff

Abstract Cancer stem cells like normal tissue stem cells have the ability to self-renew and to perform asymmetric division creating another malignant stem cell and a cell that gives rise to the phenotypically diverse tumor cell population. The hypothesis that cancer depends on a small population of cancer stem cells for its continued growth and propagation exists since Virchow proposed that cancer arises from embryo-like cells in 1858 and was further developed parallel to the development of analytical methods. To this day specific changes in cellular pathways and in the expression of surface markers have been found in leukaemia stem cells as well as in pancreatic, hepato-biliary, colon, brain, breast, ovarian, prostate, melanoma and lung cancer, nasopharyngeal tumors, laryngeal tumors and retinoblastomas as examples of solid tumors containing tumor stem cells. In cancer stem cells, developmental pathways are believed to be deregulated leading to uncontrolled self-renewal of cancer stem cells which generate tumors that are resistant to conventional therapies. Cancer stem cells as normal stem cells are likely to depend on a stem cell niche, a specialized microenvironment in which stem cells reside. Current cancer therapies aiming on tumor regression may target and kill differentiated tumor cells while sparing the rare cancer stem cell population. Cancer stem cell research is likely to provide a better insight into the mechanisms of tumor resistance to chemotherapy and might therefore lead to new therapeutic targets and better anticancer strategies.

This review will summarize the investigated surface markers of haematological and solid tumors' stem cells since the appropriate identification and isolation of this particular subpopulation of tumor cells will be essential for developing more effective cancer treatments.

Department of General and Thoracic Surgery, Division of Molecular Oncology
University Hospital Schleswig-Holstein, Campus Kiel
Arnold Heller Str. 7, D-24105 Kiel, Germany
Tel.: + 49 431 597 3432, Fax: + 49 431 597 3462
E-mail addresses
ialkatout@path.uni-kiel.de
hkalthoff@email.uni-kiel.de

T.C.G. Bosch (ed.), *Stem Cells: From Hydra to Man,*

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10.1 History and General Characterisation of Cancer Stem Cells

10.1.1 Stem Cells

Stem cells by definition have the ability to reproduce and generate new stem cells indefinitely and have the capacity to develop (differentiate) into a large number of different cell types when grown in the appropriate environment and treated with specific growth factors. Stem cells sit at the bottom of the developmental hierarchy, having the ability to self-renew and give rise to all the cell lineages in corresponding tissues. Stem cells divide to produce two daughter cells. One daughter remains a stem cell (self-renewal). The other daughter becomes a progenitor cell that undergoes expansion and further differentiation into mature cells. Stem cells have the highest potential for proliferation and a much longer life span compared with their progenies and therefore have a greater opportunity to accumulate genetic mutations. Tissue specific stem cells are distinguished from embryonic stem cells in their ability to differentiate is largely restricted to cell types within a particular organ. Although transdifferentiation (plasticity) of adult stem cells into different tissues such as the brain or blood cells into mature cells of different tissues has been reported, this apparent plasticity is often the result of a rare fusion of stem/progenitor cells of different origin. In normal tissues, homeostasis is tightly regulated to ensure the generation of mature cells throughout life without depletion of stem cell pools. Each tissue is comprised of a cellular hierarchy including stem cells able to generate all progeny, committed progenitors and terminally differentiated cells (Li and Neaves, 2006).

Pluripotent stem cells that possess both self-renewal capabilities and the ability to generate an organ-specific, differentiated repertoire of cells exist in organs other than the hematopoietic system and these can be studied to gain better insight into the stem-cell biology of a tumor. The concept of organ stem cells is difficult when one considers the many different cell types and functions of an organ, but emerging evidence indicates the existence of such pluripotent stem cells exist (Dean et al., 2005; Dontu et al., 2005).

10.1.2 Niche

Embryonic cells lose the above mentioned properties as differentiation ensues and growth-promoting signals decline. By adulthood, the remaining stem cells are dispersed and virtually invisible; however, the surviving adult stem cells have achieved to operate at a steady state. The specialized functions required to ensure proper stem cell function are vested in neighbouring differentiated cells. By signals and other

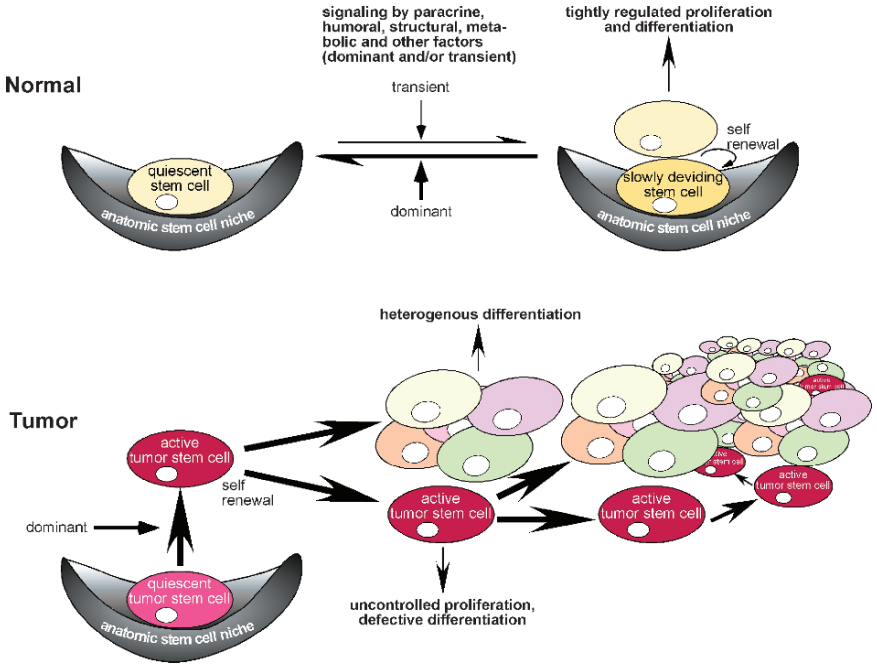

Fig. 10.1 Self renewal and differentiation of tumor stem cells generate masses of heteregenous tumor cells. Tuor growth and progression is facilitated by these relatively few active stem cells

intercellular interactions, these cells control the behaviour of adjacent stem cells that may themselves be relatively unspecialized (Fig. 10.1). In 1978 Schofield first called such regions 'niches' (Schofield, 1978). A niche is considered to be a subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production in vivo. Many niches use one or more specialized cell groups. A principal signal received by the stem cells in the niche often controls their behaviour, and may originate from within the specialized cells. Overproduction of such signals can cause stem cell hyperplasia. In all cases, the actual signalling milieu in the niche is complex and involves additional components. A basement membrane may be part of most of the niches discussed. Extracellular matrices may help to structure niches spatially and modulate the concentration of adhesive and signalling molecules locally (Spradling et al., 2001).

10.1.3 Cancer Cells Versus Cancer Stem Cells

While stem cell self-renewal is necessary for tissue repair and regeneration, it also carries the risk of genetic alteration in stem cells due to the error-prone nature of DNA replication. Deregulation of self-renewal and subsequent loss of homeostasis

may result in malignant transformation of human tissues and this forms the basis of cancer stem cell hypothesis (Li and Neaves, 2006).

One of the emerging differences between normal stem cells and cancer stem cells is their degree of dependence on the stem cell niche, a specialized microenvironment in which stem cells reside (Bjerkvig et al., 2005; Li and Neaves, 2006).

The exact origin of pluripotent stem cells in tumors might vary. They could arise from the malignant transformation of a normal stem cell that has accumulated oncogenic insults over time. Alternatively, the original tumor cell could be a more differentiated cell that develops the capacity for continuous self-renewal, thus acquiring the properties of a stem cell (Cozzio et al., 2003).

There might be numerous factors in the host microenvironment that trigger the initial steps of tumor formation (Bjerkvig et al., 2005; Li and Neaves, 2006).

The hallmarks of most cancer cells include self-sufficiency for growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis. While these features may be inherent to the majority of cells in a tumor, tumors also contain a small sub-population of cells that have characteristics of somatic stem cells capable of self-renewal, asymmetric division and multilineage differentiation (Hanahan, 2000; Neuzil et al., 2007).

In 1858 Rudolf Virchow initially proposed that cancer arises from embryonic-like cells. He suggested that cancer results from the activation of dormant embryonic-tissue remnants (Virchow, 1989). This concept, based on the histological similarities of certain types of cancer, such as teratocarcinomas, and embryonic tissues, and the observation that both tissues have an enormous capacity for both proliferation and differentiation. This hypothesis was extended by Cohnheim and Durante (Sell, 2004) to suggest that adult tissues contain embryonic remnants that normally lie dormant, but can be activated to become cancerous.

It is now half a century since bone-marrow reconstitution experiments, following lethal irradiation in mice, first indicated the existence of the haematopoietic stem cell – a cell first postulated to exist by Artur Pappenheim as early as 1917 (Jantsch, 1970; Jantsch, 1970, 1971). At around the same time that the existence of the haematopoietic stem cell was postulated, observations were reported of the heterogeneous potential of tumor cells to self-renew both in vitro and in vivo. For example, in 1973, Ernest McCulloch and colleagues observed that only 1 in 100 to 1 in 10,000 murine myeloma cells had the ability to form colonies in vitro. In 1963, Robert Bruce and colleagues showed that only 1–4% of transplanted murine lymphoma cells formed colonies in spleens of recipient animals (Bruce and Van Der Gaag, 1963). These results support the hypothesis that in the hematopoietic system, there are two different populations of multipotent progenitors – stem cells with a capacity for short-term renewal, and multipotent progenitors that cannot renew but differentiate into varied lineages in the bone marrow (Reya et al., 2001; Al-Hajj et al., 2003).

Also at this time, it was found that solid-organ cancer cells vary in their ability to proliferate in similar assays. Anne Hamburger and Sydney Salmon found that only 1 in 1,000 to 1 in 5,000 cells isolated from solid tumors were capable of forming colonies (Hamburger and Salmon, 1977). In 1961, they showed that tumor

cells that had been harvested from patients with disseminated malignancy and then injected subcutaneously into the same patients led to a low frequency of tumor formation, and that tumors were only initiated when over 1,000,000 cells were injected (Southam, 1961).

According to the cancer stem cell hypothesis, only a subpopulation of cells within a cancer has the capacity to sustain tumor growth. This subpopulation of cells is made up of cancer stem cells, which are defined simply as the population of cells within a tumor that can self-renew, differentiate, and regenerate a phenocopy of the cancer when injected in vivo (Dirks, 2006; Dirks, 2007). They are the only long-lived cells within tissues and are capable of accumulating multiple transforming mutations. These cells persist in tumors as a distinct population that likely causes disease relapse and metastasis – they are the only cells capable of, by themselves, giving rise to new tumors, in other words, they are the tumor initiating cell population e.g. under *in vivo* conditions of experimental tests like xenotransplantation in immunocompromised mice.

Evidence for cancer stem cells was first documented in haematological malignancies where only a small subset of cancer cells were capable of forming new tumors. Subsequently, cancer stem cells have been isolated from breast, brain, pancreatic, hepato-biliary, colon, ovarial, prostatic, melanoma and lung, nasopharyngeal and laryngeal cancer, retinoblastomas, including various well established cancer cell lines (Olempska et al., 2007).

For the majority of tumor entities it is still not clear whether the cancer stem cells are derived from true tissue-/organ stem cells, or bone marrow stem cells upon malignant transformation or mature cells that have undergone a de-differentiation or a transdifferentiation process.

10.1.4 Microenvironment

Examining the surroundings of tumor cells Yin et al. noticed the difference in potential of tumorigenicity by peritoneal and orthotopic injection: the intraperitoneal inoculation developed multifocal tumor quickly, but intrahepatic implantation showed a slower pattern even with increased amount of cells injection. This implicated the deciding role of the microenvironment of cancer cell propagation (Yin et al., 2007).

Developmental limitations of tissue-specific stem cells are regulated by the microenvironment. Host cells under specific conditions, such as tissue injury or infection, might provide specific signals that counteract these restrictions.

In adults, stem cells reside in a physiologically limited and specialized microenvironment, or niche, that supports stem cells but varies in nature and location depending on the tissue type. The stem cell niche is composed of a group of cells in a special location that functions to maintain stem cells. The niche is a physical anchoring site for stem cells, and adhesion molecules are involved in the interaction between stem cells and the niche and between stem cells and the extracellular

matrix. The niche generates extrinsic factors that control stem cell number, proliferation, and fate determination. Many developmental regulatory signal molecules have shown to play roles in controlling stem cell self-renewal and in regulating lineage fate in different systems. The niche controls normal asymmetrical division of stem cells. Normally, at least in the hematopoietic, intestinal, and hair follicle systems, the niche maintains stem cells primarily in a quiescent state by providing signals that inhibit cell proliferation (Zhang et al., 2003; Li and Neaves, 2006). Only upon receipt of a stimulating signal the stem cell does become activated to divide and proliferate. Therefore, stem cell proliferation depends on dynamic niche signaling. Maintaining a balance between the proliferative signal and antiproliferative signal is the key to homeostatic regulation of stem cells, allowing stem cells to undergo self-renewal while supporting ongoing tissue regeneration. Any genetic mutation that leads stem cells to become independent of growth signals, or to resist antigrowth signals, will cause the stem cells to undergo uncontrolled proliferation and possible tumorigenesis (He et al., 2005). There is a certain degree of similarity in terms of the molecules and the underlying machinery used by both normal stem cells for homing of mobilization and cancer cells for invasion and metastasis.

Zhang J. et al. found out that there are several mechanisms that can lead to changes in the hematopoietic stem cell number. An intrinsic change in stem cells that either promotes self-renewal or blocks apoptosis; an internal defect in progenitors that inhibits differentiation, leading to an accumulation of stem cells, or an external influence from the hematopoietic stem cell microenvironment. The niche size must be tightly regulated in vivo to maintain hematopoietic stem cells and normal homeostasis (Zhang et al., 2003).

Considerable progress has been made in defining the genetic elements necessary to create a tumor cell with metastatic potential. As cancers evolve, novel phenotypes molded by cumulative genetic alterations continuously emerge from the progenitor cell to adapt for survival in a given environment, whereas the cancer stem cells remain relatively stable and retain the capability for self-renewal and metastatization (Wang et al., 2006).

10.1.5 Chemotherapy

With cancer chemotherapy, some renewable tissues die off, but subsequently grow out again after treatment is stopped. These include hair, mucosal epithelium, hematopoietic cells and, unfortunately, cancer cells. That these tissues could share some fundamental functional unit was only informally considered until recent advances in stem cell biology occurred. It now seems that many tissues, both normal and malignant, contain a minority population of cells with shared functional characteristics: the ability to self-renew and to differentiate, signature characteristics of stem cells. Normal tissues contain heterogeneous types of stem cells, similarly cancer stem cells are nonhomogeneous even in a single disease such as acute myelogenous leukemia (Hope et al., 2003, 2004; Scadden, 2004).

Conventional chemotherapies might select for drugs that act specifically on the stem cells. The hypothesis suggests that conventional chemotherapies kill differentiated or differentiating cells, which form the bulk of the tumor but are unable or less able to generate new cells. A population of cancer stem cells, which gave rise to it, remains untouched and may cause a relapse of the disease.

Cancer cells can acquire resistance to chemotherapy by a range of mechanisms, including the mutation or overexpression of the drug target, inactivation of the drug, or elimination of the drug from the cell. Typically, tumors that recur after an initial response to chemotherapy are resistant to multiple drugs. Maybe one or several cells in the tumor population acquire genetic changes that confer drug resistance. These cells have a selective advantage that allows them to overtake the population of tumor cells following cancer chemotherapy. Alternatively, cancer stem cells are naturally resistant to chemotherapy through their mitotic inactivity, their capacity for DNA repair, and ABC-transporter expression. As a result, at least some of the tumor stem cells can survive chemotherapy and support regrowth of the tumor. Or, drug-resistant variants of the tumor stem cell arise, producing a population of multidrug-resistant tumor cells that can be found in many patients who have recurrence of their cancer following chemotherapy. The same mechanisms that allow stem cells to accumulate mutations over time, producing the long-term consequences of exposure to irradiation or carcinogens, would then allow cancer stem cells to accumulate mutations that confer drug resistance to their abnormally developing offspring (Reya et al., 2001).

Development of specific therapies targeted at cancer stem cells holds hope for improvement of survival and quality of life of cancer patients, especially for those suffering from metastatic disease, where little progress has been made in recent years.

10.1.6 Perspective

It remains, however, unclear whether disease recurrences originate from genetically unrelated clones differently affected by the previous treatment, or a single cancer stem clone that survives treatment and perpetuates the disease (Glinsky, 2005).

To differentiate between the diverse hypothesis it is necessary to define distinct populations of cells within tumors, based on surface immunophenotypic or functional characteristics, to purify these populations to homogeneity and to develop long-term assays of their functional ability. The specification of tumor stem cells independent of their origin can be analysed best by common surface markers. Based on functional and immunophenotypic analysis of subpopulations of cells with modern technologies, cancer has become viewed increasingly as a stem-cell disorder, in which the continued growth and propagation of the whole tumor depends on a small subpopulation of self-renewing cancer stem cells (Huntly and Gilliland, 2005a, 2005b).

10.2 Cancer Stem Cell Biological Pathways

A normal stem cell may be transformed into a cancer stem cell through genetic and/or epigenetic alterations resulting in dysregulation of the proliferation and differentiation pathways controlling it. Stem cells in their microenvironment are maintained through infrequent and mainly asymmetric divisions by which a stem cell gives rise to two daughter cells with distinct fates. Alteration of self-renewal pathways appears to be an important mechanism underlying the malignant transformation resulting n the generation of cancer stem cells.

Hedgehog is one of the signaling pathways implicated in embryonic development. It is hyperactivated in tumors and required to sustain tumor growth (Mimeault M. et al., 2007). Tumorigenic cells with characteristics similar to neural stem cells with MCM2 positivity have been isolated from paediatric brain tumors. Presence of neural stem cells could increase the aggressiveness of the original tumor (Maslov et al., 2004). *MCM2* is one of six members of the family of minichromosome maintenance proteins. MCM proteins are components of the prereplicative complex, which binds to replication origins in the G1 phase of the cell cycle and is essential for the initiation of DNA replication (http://www.ncbi.nlm.nih.gov/).

Notch is known to promote the survival and proliferation of neural stem cells through inhibition of their differentiation. Binding of ligand to a notch receptor initiates three proteolytic cleavages, two cleavages take place at the extracellular domain of Notch followed by third cleavage by a gamma-secretase complex in the plasma membrane that releases the intracellular domain of the receptor into cytoplasm (Solecki et al., 2001). This intracellular domain of Notch then translocates into nucleus to transcribe a number of target genes. Inhibitors of the gamma-secretase complex deplete stem cells and inhibit the growth of Notch-dependent tumors such as medulloblastoma and T-cell leukemia (Hallahan et al., 2004; Fan et al., 2006). Its role in control of stem cell proliferation has now been demonstrated for several cell types including haematopoietic, neural and mammary stem cells. Components of the Notch pathway have been proposed to act as oncogenes in mammary and other tumors (Dievart et al., 1999; Dontu et al., 2005).

As a tumor suppressor, *PTEN* might play a key role in maintaining the homeostasis in variety of tissues through regulating stem cell self-renewal (Li et al., 1997; Zhou et al., 2003).

The tumor suppressor *p53* has been implicated in the regulation of stem cell self-renewal. The majority of human malignancies display either p53 mutations or dysregulation of the p53 pathway (Soussi, 2005).

Wnt pathway is absolutely required for embryonic development. It regulates a number of events in cells by binding to cell-surface receptors. It is commonly hyperactivated in tumors and is required to sustain tumor growth. The Wnt pathway plays a role in self-renewal of adult stem cells and deregulation of the pathway is involved in carcinogenesis (Nusse, 2005).

10.3 Markers of Tumor Stem Cells and Their Characterization

A variety of markers have been used to characterise the tumor stem cells.

ABCG2: The membrane-associated protein encoded by this gene transports various molecules across cellular membranes. It functions as a xenobiotic transporter which may play a major role in multi-drug resistance. ABCG2 (or BCRP), an ATP-binding cassette transporter in the G2 subfamily, is a cell surface, drug-resistance marker that has been utilized to identify stem cells from a variety of tissues, including tumors and leukemias. Specifically, ABCG2 expression confers upon cells the ability to exclude Hoechst dye 33342, and confer resistance to at least 20 different chemotherapeutic agents, including methotrexate, doxorubicin, indolocarbazole, and others. The side population has been identified as a group of cells able to exclude the Hoechst 33342 dye, a characteristic feature abolished by verapamil treatment. This dye-excluding side population phenotype has been used in a variety of tissues to sort out presumptive stem cells (Seigel et al., 2005; Mohan et al., 2006).

bFGF: bFGF is a basic fibroblast growth factor which is present in basement membranes and subendothelial extracellular matrix of blood vessels. It is a factor in angiogenesis and a critical component of embryonic stem cell culture medium as it is necessary for the cells to remain in an undifferentiated state (http://www.ncbi.nlm.nih.gov, (Liu et al., 2006b)).

CD20: CD20 is a non-glycosylated phosphoprotein expressed on the surface of all mature B-cells. It is suspected that it acts as a calcium channel in the cell membrane (Polyak et al., 2003).

CD24: CD24 is a glycosylated transmembrane protein which is expressed in many B-lineage cells and on mature granulocytes. CD24 has been implicated in both activation and differentiation of B lymphocytes because its expression pattern changes at critical times during B-cell development (http://www.ncbi.nlm.nih.gov/).

CD29: CD29 is an integrin unit, which is associated with very late antigen receptors. It is expressed on resting and activated leukocytes and it is a marker for all of the very late activation antigens on cells (http://www.ncbi.nlm.nih.gov/).

CD31 (Pecam-1): CD31 plays a role in removing aged neutrophils from the body. It is normally found on endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, T/NK cells, lymphocytes, megakaryocytes, fibroblasts, neutrophils as well as in certain tumors. CD31 positive macrophages play a key role in tissue regeneration (http://www.ncbi.nlm.nih.gov/).

CD34: CD34 is a monomeric sialomucin-like adhesion molecule functioning as a cell surface antigen that is selectively expressed on human hematopoietic progenitor cells. It is a membrane protein that is produced in hematopoietic stem cells. CD34 is also expressed on endothelial capillary cells and on embryonal fibroblasts and is involved in cell adhesion. With the ongoing differentiation these cells lose the expression of CD34 (http://www.ncbi.nlm.nih.gov/), (Bonnet and Dick, 1997).

CD38: CD38 is a ADP-ribosyl cyclase novel multifunctional ectoenzyme widely expressed in cells and tissues especially in leukocytes. CD38 also

functions in cell adhesion, signal transduction and calcium signaling. It is a positive and negative regulator of cell activation and proliferation, depending on the cellular environment. It is expressed at variable levels on the majority of hematopoietic cells, prevalently during early differentiation and activation (Deaglio et al., 2001).

CD41 (Integrina2b): CD41 is a receptor for fibronectin, fibrinogen, plasminogen, prothrombin, thrombospondin and vitronectin. Following activation CD41 brings about platelet interaction through binding of soluble fibrinogen. This step leads to rapid platelet aggregation with physically plugs ruptured endothelial cell surface (Larson and Springer, 1990).

CD44: The CD44 protein is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. This protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis. CD44 mediates cell-cell and cell-matrix interactions. Adhesion with hyaluronic acid plays an important role in cell migration, tumor growth and progression (http://www.ncbi.nlm.nih.gov/projects/mhc/).

CD45: CD45 is a type I transmembrane protein which is present on all differentiated hematopoietic cells except erythrocytes and assists in the activation of those cells (co-stimulation) (http://www.ncbi.nlm.nih.gov/).

CD90 (Thy-1): CD90 is a anchored conserved cell surface protein. It can be used as a marker for stem cells and for axonal processes of neurons. And it has been proposed to play a role in cell-cell and cell-matrix interactions (Kemshead et al., 1982).

CD105 (Endoglin): CD105 is a type I membrane glycoprotein located on cell surfaces and is part of the TGF beta receptor complex. It has been found on endothelial cells, activated macrophages, fibroblasts, and smooth muscle cells. It might be involved in cytoskeletal organization affecting cell morphology and migration (Sanz-Rodriguez et al., 2004).

CD117: CD117 cytokine receptor is expressed on the surface of hematopoietic stem cells and other cell types. It identifies certain types of hematopoietic progenitors in the bone marrow. It shows high level expression in hematopoietic stem cells, multipotent progenitors, and myeloid progenitors. Mutations of CD117 can lead to cancer (http://atlasgeneticsoncology.org/Genes/KITID127.html).

CD133 (prom1, prominin1): The CD133 gene codes for a pentaspan transmembrane glycoprotein. It is expressed in a subset of stem progenitor cells in the hematopoietic system as well as in solid tumors of the brain, prostate, colon, pancreas and liver and it is a commonly used stem cell marker. The CD133 antigen is recognized by three monoclonal antibodies. These antibodies recognize two different epitopes CD133/1 and CD133/2, respectively. These epitopes do not relate to the two splicing forms of CD133 mRNA. While tumor-stem cells specific for various tissues differ in their phenotype, various tumor-stem cells, in particular those of the epithelial origin, share a unifying phenotype with high levels of CD133 expression. High levels of CD133 expression is now accepted as an important marker for a number of different cell lineages. CD133 + tumor-stem cells are resistant to therapy, consistent with their stem-like nature as reported both for chemotherapy and radiation

treatment. However, since the function of CD133 is unknown, it is not clear if CD133 is just a marker of resistant cells or whether high expression of CD133 in tumor-stem cells could contribute to the resistance to therapy (Yu et al., 2002; Neuzil et al., 2007).

CD138 (Syndecan-1): CD138 is a transmembrane heparan sulfate proteoglycan macromolecule. It interacts with extracellular matrix proteins. It is expressed on normal and malignant human plasma cells and on basolateral surfaces of endothelial cells but not on virgin/naive B cells, memory B cells, T cells, or monocytes (http://www.ncbi.nlm.nih.gov/).

CD166: Activated leukocyte adhesion molecule (CD166) is a member of the immunoglobulin super family and is a type 1 transmembrane protein. CD166 is expressed on the surface of mesenchymal stem cells and has been found on human melanoma cell lines. Its expression correlates with tumor thickness in primary melanoma (Swart et al., 2005).

CK18: CK18 is a constituent of the hepatocyte cytoskeleton. It plays a role in the detection of all simple epithelial tissues, as found in liver, pancreas, and intestine. It is not staining squamous cells and is used to differentiate carcinomas and lymphomas (Miki et al., 2007).

CK19: CK19 is a type I keratin that constitutes the Type I intermediate filaments of the intracytoplasmatic cytoskeleton (Wang et al., 2007).

CK134 (34bE12): This antibody is a marker of squamous epithelia and complex epithelials in normal tissues, e.g. basal cell in acini of the prostatic gland. In tumors CK134 has a positive staining of squamous cell carcinoma whereas adenocarcinomas are not stained (Bratthauer et al., 2003).

c-Met: This protein is expressed mostly in epithelial cells, but also in endothelial cells, neural cells, hepatocytes, hematopoietic cells, melanocytes. It is the membrane receptor for HGF. Its activation plays a key role in cellular physiology: mitogenesis, motogenesis, morphogenesis (Peruzzi and Bottaro, 2006).

CXCR4 (Fusin): CXCR4 is an alpha-chemokine receptor for SDF-1, a molecule endowed with potent chemotactic activity for lymphocytes. The ligand is important in hematopoietic stem cell homing and hematopoietic stem cell quiescence (http://www.ncbi.nlm.nih.gov/).

Cx43: Connexins are a family of structurally related transmembrane proteins that assemble to form vertebrate gap junctions which are essential for coordinated depolarization of cardiac muscle, and proper embryonic development. Cx43 is expressed at the surface of vasculature with atherosclerotic plaque, between granulosa cells, which is required for proliferation. It is normally expressed in astrocytes (Schiavi et al., 1999).

ESA (Flotillin 2): Caveolae are small domains on the inner cell membrane involved in vesicular trafficking and signal transduction. Flotillin 2 encodes a caveolae-associated, integral membrane protein. Flotillin 2 is thought to function in neuronal signaling (Shin et al., 2003).

gp100: This antigen is associated with melanosomes (http://www.ncbi.nlm.nih.gov).

Nerve growth factor: NGF is a small secreted protein which induces the differentiation and survival of particular target neurons. It is critical for the survival and maintenance of sympathic and sensory neurons (Ibanez, 2002).

LY6E: A monoclonal antibody recognizes this 20-kD glycoprotein which is present in desmosomal intercellular adhesions of keratinocytes and in squamous carcinoma cells (http://www.ncbi.nlm.nih.gov/).

Nestin: Nestin is an intermediate filament expressed in the cytoplasm of neuroepithelial stem cells. Its expression has also been found in metastatic melanomas (http://www.ncbi.nlm.nih.gov/).

Oct-4 (Octamer-4): Is a homeodomain transcription factor and is critically involved in the self-renewal of undifferentiated embryonic stem cells. Oct-4 expression must be closely regulated; too much or too little will actually cause differentiation of the cells. It is expressed in developing embryos throughout the preimplantation period. This transcription factor is initially active as a maternal factor in the oocyte but remains active in embryos throughout the preimplantation period. Oct-4 expression is associated with an undifferentiated phenotype and tumors. In fact gene knockdown of Oct-4 promotes differentiation, thereby demonstrating a role for these factors in human embryonic stem cell self-renewal (Niwa et al., 2000).

PDGF: Platelet-Derived Growth Factor is a protein that regulates cell growth and division. It plays a significant role in angiogenesis. It also plays a role in embryonic development, cell proliferation, cell migration. PDGF is linked to atherosclerosis, fibrosis and malignant diseases and it allows a cell to skip the G1 checkpoints in order to divide (McClintock et al., 1992).

TACSTD1: TACSTD1 encodes a carcinoma-associated antigen and includes at least two type I membrane proteins. This antigen is expressed in most normal epithelial cells and gastrointestinal carcinomas and functions as a homotypic calcium-independent cell adhesion molecule (http://www.genecards.org/cgi-bin/).

10.4 Hematopoietic and Solid Tumors and So Far Analysed Surface Markers (See Table 10.1)

While stem cell self-renewal is necessary for tissue repair and regeneration, it also carries the risk of genetic alteration in stem cells due to the error-prone nature of DNA replication. Thus the pathways that control stem cell self-renewal and the microenvironment in which the stem cells reside both may play a role in carcinogenesis (Rossi and Weissman, 2006). Deregulation of self-renewal and subsequent loss of homeostasis may result in malignant transformation of human tissues and this forms the basis of cancer stem cell hypothesis.

Leukemia: Increasing evidence shows that the AML leukemic clone is organized as a hierarchy of hematopoietic stem cells and committed progenitor cells with many similarities to the normal hematopoietic system has provided fresh insight into the mechanisms that initiate and maintain the myeloid leukemias. Evidence for

Table 10.1 Surface markers

Marker	Studies positive	Expression	No expression
ABCG2	5/5	Pancreas cancer (Olempska et al., 2007) Hepato-biliary cancer (Zen et al., 2007) Lung cancer (Ho et al., 2007) Retinoblastoma (2 times) (Seigel et al., 2005; Mohan et al., 2006)	
bFGF	1/1	Brain tumors (Kondo et al., 2004)	
CD20	2/2	Breast cancer (Al-Hajj et al., 2003) Melanoma (Fang et al., 2005)	
CD24	1/6	Pancreas cancer (Li et al., 2007)	Breast cancer (3 times) (Al-Hajj et al., 2003; Abraham et al., 2005; Ponti et al., 2005) Lung cancer (Ho et al., 2007) Ovarian cancer (Szotek et al., 2006)
CD29	1/1	Hepato-biliary carcinoma (some cell lines) (Suetsugu et al., 2006)	
CD31	0/2		Ovarian cancer (Szotek et al., 2006) Lung cancer (Kim et al., 2005)
CD34	3/8	Leukemia (2 times) (Lapidot et al., 1994; Bonnet and Dick, 1997) Hepato-biliary carcinoma (some cell lines) (Suetsugu et al., 2006)	Leukemia (Wulf et al., 2001) Pancreas carcinoma (Gou et al., 2007) Ovarian cancer (Szotek et al., 2006) Lung cancer (2 times) (Kim et al., 2005; Ho et al., 2007)
CD38	0/2		Leukemia (2 times) (Lapidot et al., 1994; Bonnet and Dick, 1997)
CD41	3/3	Prostate cancer (3 times) (Richardson et al., 2004; Collins et al., 2005; Miki et al., 2007)	
CD44	11/13	Pancreas carcinoma (2 times) (Gou et al., 2007; Li et al., 2007) Hepato-biliary carcinoma (some cell lines) (Suetsugu et al., 2006) Brain tumors (Liu et al., 2006a) Breast cancer (3 times) (Al-Hajj et al., 2003; Abraham et al., 2005; Ponti et al., 2005)	Ovarian cancer (Szotek et al., 2006) Lung cancer (Ho et al., 2007)

(continued)

Table 10.1 (continued)

Marker	Studies positive	Expression	No expression
		Prostate cancer (4 times) (Collins et al., 2005; Patrawala et al., 2006; Gu et al., 2007; Miki et al., 2007)	
CD45	0/2		Ovarian cancer (Szotek et al., 2006) Lung cancer (Kim et al., 2005)
CD90	2/2	Brain tumors (Liu et al., 2006a) Retinoblastoma (Seigel et al., 2007)	
CD105	0/1		Ovarian cancer (Szotek et al., 2006)
CD117	2/2	Hepato-biliary carcinoma (some cell lines) (Suetsugu et al., 2006) Ovarian cancer (Szotek et al., 2006)	
CD133	16/17	Pancreas cancer (Olempska et al., 2007) Hepato-biliary carcinoma (3 times) (Suetsugu et al., 2006; Ma et al., 2007; Yin et al., 2007) Colon carcinoma (2 times) (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) Brain tumors (4 times) (Hemmati et al., 2003; Singh et al., 2003, 2004; Liu et al., 2006a) Prostate cancer (3 times) (Richardson et al., 2004; Collins et al., 2005; Miki et al., 2007) Larynx carcinoma (Zhou et al., 2007) Retinoblastoma (Seigel et al., 2007) Melanoma (Klein et al., 2007)	Ovarian cancer (Szotek et al., 2006)
CD138	4/5	Prostate cancer (2 times) (Lawson et al., 2005; Xin et al., 2005) Lung cancer (Kim et al., 2005) Retinoblastoma (Seigel et al., 2005)	Ovarian cancer (Szotek et al., 2006)
CD166	1/1	Melanoma (Klein et al., 2007)	
CK18	1/2	Prostate cancer (Miki et al., 2007)	Breast cancer (Ponti et al., 2005)
CK19	1/1	Nasopharyngeal carcinoma (Wang et al., 2007)	
CK134	1/1	Prostate cancer (Miki et al., 2007)	
c-Met	2/3	Breast cancer (Al-Hajj et al., 2003) Melanoma (Fang et al., 2005)	Pancreas carcinoma (Gou et al., 2007)
CXCR4	1/1	Brain tumors (Liu et al., 2006a)	
Cx43	0/1		Breast cancer (Ponti et al., 2005)

(continued)

Table 10.1 (continued)

Marker	Studies positive	Expression	No expression
ESA	2/3	Pancreas cancer (Li et al., 2007)	Breast cancer (Ponti et al., 2005)
		Breast cancer (Al-Hajj et al., 2003)	
gp100	1/1	Melanoma (Grichnik et al., 2006)	
Nerve growth factor	0/1		Melanoma (Grichnik et al., 2006)
LY6E	1/1	Pancreas cancer (Gou et al., 2007)	
Nestin	7/8	Brain Tumors (3 times) (Hemmati et al., 2003; Singh et al., 2004; Liu et al., 2006a)	Lung cancer (Ho et al., 2007)
		Prostate cancer (Gu et al., 2007)	
		Retinoblastoma (Seigel et al., 2007)	
		Melanoma (2 times) (Grichnik et al., 2006; Klein et al., 2007)	
Oct-4	6/6	Brain tumors (Liu et al., 2006a)	
		Breast cancer (2 times) (Al-Hajj et al., 2003; Ponti et al., 2005)	
		Prostate cancer (2 times) (Patrawala et al., 2006; Gu et al., 2007)	
		Retinoblastoma (Seigel et al., 2007)	
PDGF	1/1	Brain tumors (Kondo et al., 2004)	
TACSTD1	1/1	Pancreas carcinoma (Gou et al., 2007)	

cancer stem cells was first (1997) documented in hematological malignancies where only a small subset of cancer cells were capable of forming new tumors. Using in vivo models where human acute myeloid leukaemia (AML) cells were transplanted into immunodeficient mice, a leukaemia-initiating cell was identified that showed strikingly immature features, expressing a CD34 + /CD38- phenotype (Lapidot et al., 1994; Bonnet and Dick, 1997).

Wulf et al. suggested that CD34-hematopoietic stem cells may be more primitive than cells expressing the CD34 antigen and, when transformed, could underlie some cases of AML. They demonstrated the involvement of CD34low/-side population cells in a substantial percentage of AML cases. (Wulf et al., 2001).

Similar techniques have been used to demonstrate a heterogeneous expression of cellular immunological markers in solid tumors including breast, prostate, brain, colon, skin (melanoma), pancreas, hepato-biliary carcinoma, ovary, laryngeal, nasopharyngeal, retinoblastoma and lung cancer. Implantation of small subsets of such solid tumors revealed that only the cells with stem cell characteristics were able to form tumors suggesting the existence of cancer stem cells in these tumors.

Due to these results research on different solid tumors described a broad variety of surface markers in vitro and in vivo. Different projects showed the expression/

non-expression of markers by immuno staining of tissues, cell-lines, and in xenografts in immunodeficient mice. There are about 42 studies that analysed the expression of one or several surface markers of the different solid tumors up to date (see Table 10.1).

These studies suggest that several stem cell markers may be shared by cancer stem cells in different tumor types, such as CD44 and CD133. However, it is possible that each tumor has its own unique phenotype for markers, as highly tumorigenic breast cancer cells are CD24-, whereas their pancreatic counterparts are CD24 + (Al-Hajj et al., 2003; Abraham et al., 2005; Ponti et al., 2005; Li et al., 2007). It is difficult to identify cancer stem cells, because they are usually present in very small numbers and specific surface markers are still unknown. Moreover, technical hurdles are to be overcome in stem cell as well as in tumor stem cell research. A whole genome DNA microarray analysis may be useful for identifying potential candidates for specific cancer stem cell markers or even for defining a definitive cancer stem cell pattern. If cancer stem cell specific surface markers are confirmed, this will allow appropriate isolation of this important population of cells and cancer therapy and science will be revolutionized (Haraguchi et al., 2006). Yet, we still do not know whether all cancers are organized into a hierarchy of cells with different proliferative and differentiation potentials (Seigel et al., 2005). It is proposed that tumor stem cells give rise to transiently amplifying tumor cells that make up the majority of the tumor bulk before terminally differentiating. Theoretically, as long as a nidus of tumor stem cells are present, the tumor can continue to expand, and if the tumor stem cells circulate, they can give rise to distant metastasis (Grichnik, 2006).

10.5 Future Perspectives

The lack of substantial progress in treating a variety of common advanced human cancers suggests that a change in our current strategy is needed. In addition to drug resistance, the recurrence of tumors after initial regression by conventional therapies is also very frequent. Cancer cells can acquire resistance to chemotherapy by a range of mechanisms, including the mutation or overexpression of the drug target, inactivation of the drug, or elimination of the drug from the cell. Based on the tumor stem-cell concept, an alternative model proposes that the cancer stem cells are naturally resistant to chemotherapy through their quiescence, their capacity for DNA repair, and ABC-transporter expression. As a result, at least some of the tumor stem cells can survive chemotherapy and support regrowth of the tumor. Another model of acquired resistance suggests that drug-resistant variants of the tumor stem cell or its close descendants arise, producing a population of multidrug-resistant tumor cells that can be found in many patients who have recurrence of their cancer following chemotherapy (Dean et al., 2005; Dontu et al., 2005). The rapid relapse observed with some tumors, at times within one cycle of chemotherapy, finds a parallel setting in the repopulation of the bone

marrow by normal hematopoietic stem cells and the recovery of the mucosal layers of the gastrointestinal tract, both of which usually occur within a 3-week cycle. Similarly, tumor recurrences that occur months to years after an original response to chemotherapy can be modelled on the slower recovery that is observed with hair follicles (Cotsarelis and Millar, 2001).

Unfortunately, for some highly drug-resistant cancers, including kidney, pancreatic cancer, and glioblastomas, the problem is not that a few cells survive but, rather, that only a minor fraction of cells directly die in response to chemotherapy. But at least for cancers, that respond to chemotherapy with an apparent clinical complete response, only to relapse months or years later, this stem-cell model of drug resistance has a great appeal (Cisternino et al., 2004a, 2004b).

Design and development of new cancer treatments is therefore necessary to target stem cell properties; self-renewal and differentiation. If the malignancy is a result of a blocked ontogeny then it should be possible to treat cancer by inducing differentiation of cancer cells potentially including cancer stem cells which would loose their self-renewal property.

In addition to induce differentiation, a number of stem cell self-renewal pathways have been targeted for treatment of various human tumors. Taken together, therapeutic strategies aimed either inducing differentiation or death of cancer stem cells and this may lead to more effective cancer therapies.

Because there is a considerable congruence between normal and malignant stem-cell function, it is probable that if we understand one, we will have major insights into the other. Nonetheless, the discovery of key signalling pathways that underlie the common ground of plasticity of embryonic stem cells and multipotent tumor cells might yield new therapeutic strategies to suppress the metastatic phenotype (Hendrix et al., 2007).

As shown by Yin S. et al. the intraperitoneal inoculation of CD133 + hepatic tumor stem cells developed multifocal tumor quickly, but intrahepatic implantation showed a slower pattern even with increased amount of cells injection. This findings points to the crucial role of the microenvironment for cancer cell propagation and this must be taken into consideration for assessment of tumorigenicity in vivo. There might be numerous factors in the host microenvironment that trigger the initial steps of tumor formation (Bjerkvig et al., 2005; Yin et al., 2007) providing novel therapeutic options in oncology based on a rational and thorough understanding of this fundamental cell biological process.

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