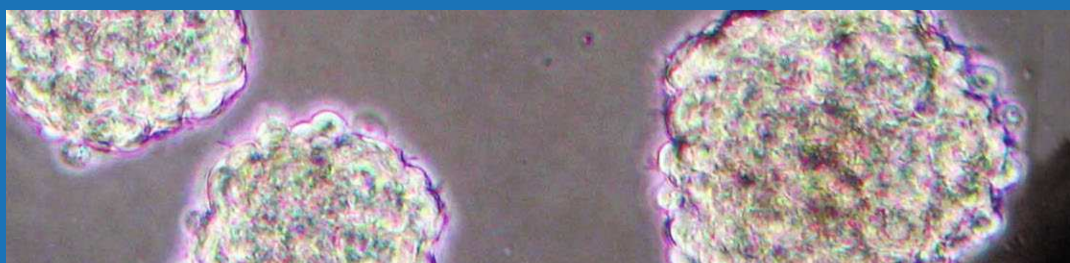
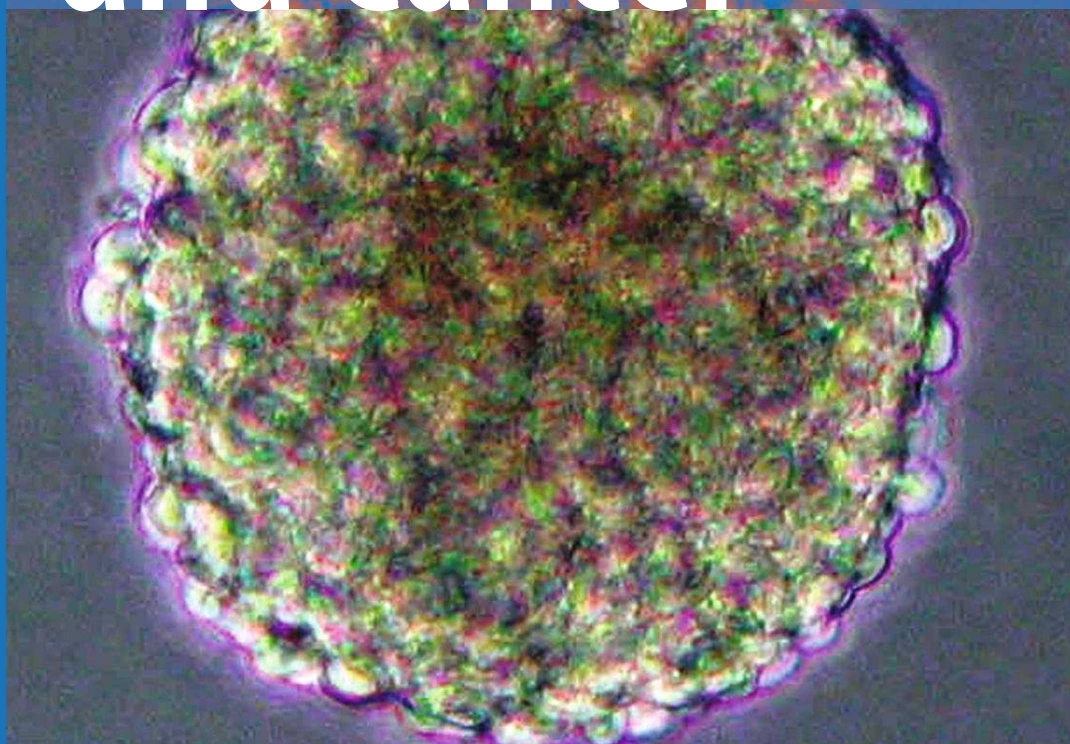
Sadhan Majumder
Editor



Stem Cells and Cancer



Stem Cells and Cancer

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Editor

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Introduction to Stem Cells and Cancer

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

T. S. Eliot, *Four Quartets*

The concept of a stem cell is not new to the biologist or medical researcher. We keep coming back to this concept, periodically, each time with a renewed sophistication based on advances in knowledge and technology. But for scientists, unlike Eliot, the cycle of exploration never ends.

The cell theory developed in the early 1800s was formally applied to the problem of cancer by Johannes Muller and his pupil, Rudolf Virchow, who proposed that cancer arises from embryo-like cells in 1855 (Haggard and Smith 1938; Virchow 1855).

It was a century later that Till and McCulloch (1961) described a technique for detecting a small population of hematopoietic cells present in murine marrow which could form colonies in the spleen when injected into irradiated mice. These cells were shown to have the capacity both to self-renew and to differentiate into mature hematopoietic cells – dual qualities that define a stem cell.

The clonality of these cells which could form colonies in the spleen was soon established using genetic techniques (Wu et al. 1967). Methods for studying colony-forming hematopoietic cells were extended to in vitro cell culture by Bradley and Metcalf (1966), greatly enhancing the capacity to study cells with stem cell properties. This approach was extended to cancer cells, culminating in a general assay reported by Hamburger and Salmon (1977). In the meantime, the clonality of human cancer had been established through studies of genetic markers in human cancer cell populations (Fialkow 1974).

A seminal publication by Nowell in 1977 brought together evidence from a variety of sources and raised the hypothesis that the malignant clone from which a cancer originates may change in its capacity to grow and metastasize as a result of accumulated genetic mutations, which produce cancer cells that dominate the tumor cell population through a natural selection process (Nowell 1976).

During the next few decades cell surface markers for identifying stem cells were discovered, and separation methods using flow cytometry enabled collection of purified stem cells in quantities that could be studied. In 1994 Dick and his colleagues formally demonstrated that murine acute myeloid leukemias contain a minority population of cells with stem cell markers and properties, which can be propagated when transferred to other mice and can generate a leukemic condition (Lapidot et al. 1994; Hope et al. 2004). This was the first formal demonstration that the hierarchical organization of stem cells and differentiated cells observed in the normal hematopoietic system also characterizes the organization of cancer cells within a tumor.

This observation was followed nearly a decade later by Clarke's demonstration of stem cells in a subpopulation isolated by flow cytometry from human breast cancers (Al-Hajj et al. 2003). After this, a sequence of studies rapidly demonstrated the presence of stem cells in a variety of human cancers (Journal of Clinical Oncology, 2008). Many of these discoveries are reported and elaborated upon in this volume.

Today, the evidence from many experts supports the concept that the differentiated cells in normal tissues originate from stem cells, and the concept that a malignant tumor originates from a cell with cancer stem cell qualities. What remains to be understood is whether cancer stem cells are derived from normal tissue stem cells, or whether mutations and altered gene expression in more differentiated cells can result in generation of a cancer cell with stem cell capabilities, or both.

The latter model entails the view that differentiated cells have plasticity which enables them to assume stem cell characteristics if appropriate genes are expressed. Two lines of recent research findings provide evidence that this model of the genesis of cancer stem cells is worthy of serious consideration.

Recently several laboratories have demonstrated that introduction of just four active genes into a mature differentiated cell can convert it into a cell with embryonic stem cell characteristics (Takahashi and Yamanak 2006; Takahashi et al. 2007; Yu et al. 2007).

Even more intriguing from the viewpoint of this volume are recent studies exploring the transitions that occur in cancer cells which are undergoing the process of invading and metastasizing from a primary site to a distant site in the patient. In this case, cancer cells with some differentiated characteristics which identify them as arising in a particular epithelial tissue (e.g., colon cancer) undergo an epithelial–mesenchymal transition (EMT) which arms them with new characteristics that include the capacity to invade other tissues and metastasize. These cells, with acquired mesenchymal cell capabilities, have been shown to display markers and characteristics of stem cells (e.g., CD44) and to lose markers and characteristics of epithelial cells. Once they have reached a hospitable environment which provides the necessary growth-promoting molecules and no longer produces EMT-inducing signals, these malignant cells with stem cell characteristics undergo a reverse mesenchymal–epithelial transition and form a new mass of cancer cells which have the formerly displayed epithelial characteristics.

This demonstration of plasticity suggests the possibility that a cancer cell can assume and discard stem cell-like characteristics, and that the products of normal cells in the environment may contribute to this process (Weinberg 2007; Mani et al. 2008).

Thus the model of cancer progression through accumulated genetic mutations and a process involving selection of the fittest cells that can spread and metastasize may be modified to create a new model, in which phenotypic plasticity enables reversible alterations in the expression of genes critical for invasion and metastasis.

These recent discoveries have tremendous implications for our understanding of the process of malignant transformation and for our approach to devising mechanism-based treatments for cancer. Cancer cells with stem cell-like qualities (which include a relative resistance to chemotherapy and radiation therapy) must be targeted in our development of new anti-cancer therapies.

The model of cancer stem cells outlined above is recent and undoubtedly will undergo modification as further knowledge accumulates. There are fundamental differences between cells that produce normal tissues and cancer cells with stem cell-like qualities, which need to be better understood. And it remains to be determined whether the latter are derived from the cells that produce normal tissues. This book brings together outstanding international experts explaining our current understanding of basic principles of cancer stem cells. This book provides timely, cutting-edge information about cancer stem cells from the perspectives of both the basic and clinical sciences and will help researchers move with greater speed towards designing more effective treatments for cancer.

Urgency derives from the human cost of cancer. Since cancer will occur in over 40% of Americans and the death rate from cancer is over 500,000 per year, cancer stem cells are a subject worthy of intensive research by basic, translational, and clinical investigators whose goal is to improve these statistics.

And so scientists continue to revisit the concept of stem cells, now with nuances that include plasticity and more clearly defined interactions between the cell and its environment, but with many open questions demanding further exploration.

We dance round in a ring and suppose,
But the Secret sits in the middle and knows.
Robert Frost, *The Secret Sits*

Houston, USA John Mendelsohn

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Stem Cells and Cancer: An Introduction

Stewart Sell

Abstract Cancer tissue contains the same cell populations as do normal adult tissues: stem cells, proliferating transit-amplifying cells, terminally differentiated (mature cells) and dead cells. However, the cancer transit-amplifying cells are arrested at a stage of maturation where many of the transit-amplifying cells continue to divide and do not die. During normal tissue renewal, the transit-amplifying cells produce progeny that differentiate and die. Because of this difference, cells in a cancer continue to accumulate. On the other hand, the number of dividing cells in normal tissue essentially equals the number of differentiating cells, so that the total number of cells remains relatively constant.

The idea that cancer arises from stem cells was first proposed over 150 years ago as the embryonal rest theory of cancer. However, by the beginning of the 20 th century, the embryonal rest theory of cancer was discarded, and the hypothesis that cancer arises from de-differentiation became generally accepted. Then, about 50 years ago, studies on cancers of germinal cells (teratocarcinomas) re-established the principles that cancer arises from stem cells, that cancers contain stem cells, and that cancer could be treated by induction of differentiation (differentiation therapy). However, teratocarcinomas were considered exceptions to the rule, and the de-differentiation theory of origin remained generally accepted for most cancers until the 1980 s. Then studies on the cellular origin of cancer during experimental chemical hepatocarcinogenesis showed that hepatocellular cancer did not arise from de-differentiation of hepatocytes, as was generally believed, but rather from maturation arrest of cell in the hepatocyte lineage. The re-emergence of the stem cell theory of cancer preceded the current excitement in cancer stem cells.

Over the last 10 years, differentiation therapy has been applied with great success to cancer of the blood cells (leukemias) by inactivation of the signaling pathways that allow the leukemic transit-amplifying cells to continue to

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proliferate and not die (maturation arrest). Differentiation therapy of cancer stem cells is now proposed through the use of small inhibitory molecules or inhibitory RNAs (iRNAs) to block the signals that maintain “stemness” so that the leukemic stem cells are allowed to differentiate. Conventional chemotherapy, radiotherapy, and anti-angiogenic therapies act on the cancer transit-amplifying cells. When these therapies are discontinued, the cancer will re-form from the therapy-resistant cancer stem cells. Successful differentiation therapy of cancer stem cells would force these cells to differentiate, so that they can no longer re-establish the cancer.

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1 Stem Cell Origin of Cancer

The cell of origin of all tissues is called a stem cell. From this cell all other cells arise. The fertilized ovum is the primordial stem cell for all of the cells of the human body. The immediate progeny of the primordial stem cells are embryonic stem cells, which, in turn, give rise to tissue stem cells. It is from these tissue stem cells that most cancers arise.

1.1 Cancer Is a Problem of Developmental Biology

Barry Pierce and his colleagues stated in their book in 1978 that cancer is a problem of developmental biology (Pierce et al., 1978). For almost 200 years pathologists and cell biologists have noted similarities between cancer and embryonic development (Pierce et al., 1978; Sell, 2004a,b; Sell and Pierce,

1994; see embryonal rest theory of cancer later). In simplistic terms, the cellular processes that occur during embryonal development consist of two phases: expansion and determination (Fig. 1A). Following fertilization of the ovum, the primordial embryonal stem cell divides symmetrically for the first 5–6 divisions. During these symmetric divisions (Fig. 1B), each embryonal cell produces two daughter cells, which are also able to divide. Thus, the first phase of embryonal development results in exponential expansion of the embryonal cells (Sell, 2004b). The primordial stem cell, the fertilized egg, and its embryonic stem cell progeny are totipotent; they have the potential to produce progeny for all embryonic and adult tissues. During the second phase of development,

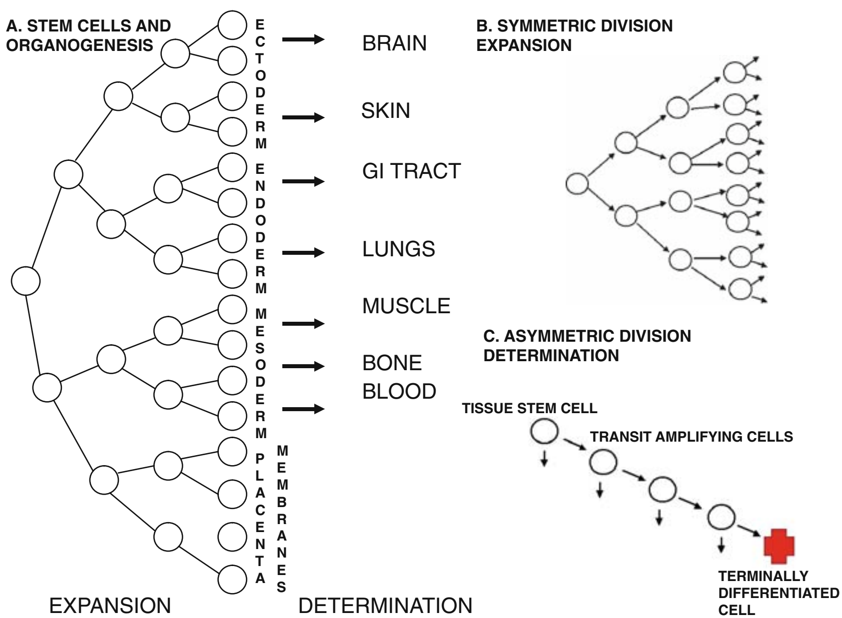

Fig. 1 Proliferation and determination of embryonic stem cells during early development. **A** Embryonic stem cells and organogenesis; **B** symmetric division (expansion); **C** asymmetric division (determination). During the first stage of embryogenesis, the primordial stem cell and each embryonic stem cell divide, to produce two equivalent daughter cells each (symmetric division). This results in an exponential expansion of the number of cells in the embryo. Then, the type of cell division changes to asymmetric. During asymmetric division, one daughter cell remains a stem cell, whereas the other daughter cell begins the process of differentiation, to produce mature cells that carry out the specialized function mature organs. In adult tissue, normal tissue renewal is carried out through asymmetric division of tissue-specific transit-amplifying cells. The number of transit-amplifying cells that divide is equal to the number that differentiates, so the number of cells in adult tissue remains constant. However, in cancer tissues some of the transit-amplifying cells divide symmetrically, cell maturation is delayed, and cancer cells increase in number

determination, the type of division changes to asymmetrical (Fig. 1C). During asymmetric division one daughter cell remains a stem cell, while the second daughter cell begins the process of differentiation into the mature tissue cells of the developing fetus. In this way, the progeny of the totipotent embryonic stem cells become tissue stem cells with more limited potential than embryonic cells, and branches (lines) of cells are produced that form various differentiated organs. Tissue-determined stem cells are retained during development and persist into adult tissues, where they serve as reserve stem cells for normal tissue renewal (Sell, 2004a,b). Cancer as a cellular process is caused by a failure of the cells in the adult tissues to mature normally (maturation arrest; Sell and Pierce, 1994), so that instead of terminally differentiating to mature tissue cells, they retain the proliferation potential of embryonic transit-amplifying cells.

1.1.1 Normal Tissue Renewal and Cancer

Normal tissue and cancer tissue contain the same populations of cells: stem cells, transit-amplifying cells, and terminally differentiated cells (Pierce et al., 1978; Sell, 2004a,b, 2006a; Sell and Pierce, 1994; Reya et al., 2001). Normal tissue renewal and growth of cancer are both accomplished by division of the transit-amplifying cells (Fig. 1C). Usually, the stem cells of both normal tissue and cancers are relatively few in number, compared to the transit-amplifying cells and the terminally differentiated cells, and they do not participate in proliferation. The proliferating cells of both cancers and normal tissue are the transit-amplifying cells. Cancer tissue differs from normal tissue in that the transit-amplifying cells accumulate in cancer, whereas cells in normal tissue differentiate so that they no longer divide (terminal differentiation).

1.1.2 Skin Cell Renewal

One of the best examples of the normal cellular lineage and also of the contribution of maturation arrest to cancer is skin. The pluripotent skin epidermal stem cells are located in the bulb of the hair follicle (Perez-Losada and Balmain, 2003). The epidermis-committed stem cells are located in the basal layer of the skin (germinativum) and are much fewer in number than the transit-amplifying cells located in the spinosum layer. Maturation is accomplished through the accumulation of cytokeratin, which becomes prominent in the granular layer. The granules contain cytokeratin. The cytoplasm of the cells in the granular layer becomes filled with these granules and eventually the cells lose their structure, forming the outer layer of acellular keratin, known as the corneum.

1.1.3 Skin Cancer

Skin cancers arise by maturation arrest at various levels of differentiation of the epidermis (Fig. 2A; Perez-Losada and Balmain, 2003; Owens and Watt, 2003). Maturation arrest of the primitive skin progenitor cells in the bulge of the hair

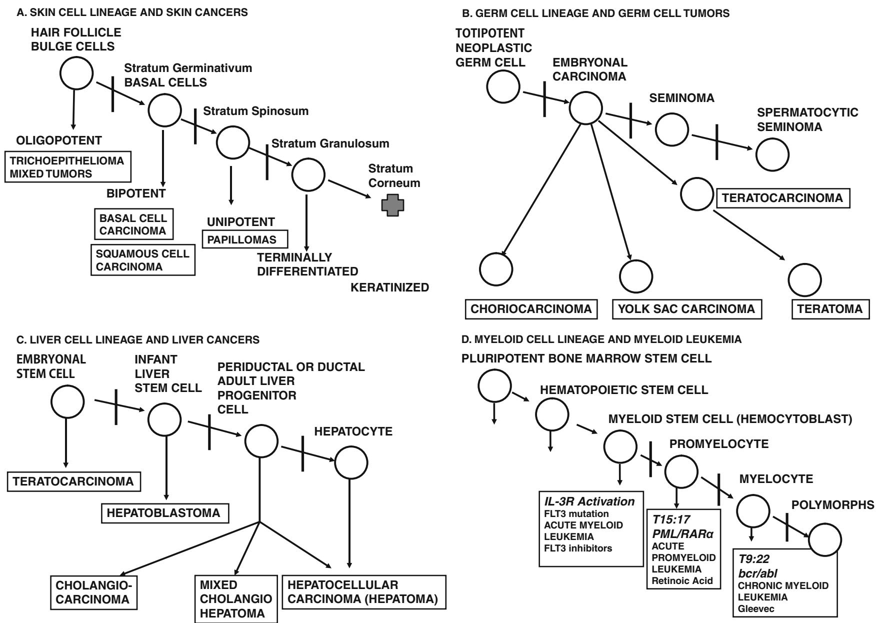

Fig. 2 Selected cell lineages and stage of maturation arrest for various cancers. **A** Skin cell lineage and skin cancers. The type of skin cancer is related to the stage of differentiation at which a block in maturation occurs. The vertical lines indicate a block in maturation. If the block takes place at the level of the stem cells in the bulge of the hair follicle, mixed tumors of hair follicle, sweat gland, and sebaceous gland elements will form; if the block occurs at the level of the basal stem cell, basal or squamous cell cancers form; if the block is at the level of the stratum spinosum, benign papillomas form. **B** Germ cell lineage and germ cell tumors. The figure depicts the stages of maturation of germ cells at which maturation arrest produces various tumors of the germ cell lineage. Embryonal carcinoma is a tumor of the totipotent embryonal stem cells. From these are derived tumors that contain elements of embryonal carcinoma, including choriocarcinoma (placenta), yolk sac tumors, and teratocarcinomas. Teratomas are benign tumors of mixed mature cells. Seminomas are tumors expressing the spermatocytic lineage of germinal cells. **C** Liver cell lineage and liver cancers. Depicted is a lineage of cells: embryonal stem cells, infant liver cells, adult liver progenitor cells, and hepatocytes. Maturation arrest at the embryonal cell level produces embryonal cell carcinomas or teratocarcinomas. Hepatoblastomas occur only in children under the age of 4–5 years and are considered to arise from a hepatic progenitor cell that disappears after this age. In the adult, liver tumors of bile duct cells (cholangiocarcinomas) and hepatocellular carcinomas arise from a "bipolar" progenitor cell in the adult liver. Hepatocellular cancinomas can also arise from mature hepatocytes, since there are essentially no terminally differentiated cells in the hepatocytic lineage. From Sell (2006b). **D** Gene translocations, levels of maturation arrest, and differentiation therapy of selected human leukemias. Specific gene translocations lead to expression of signaling molecules that constitutively activate cells at various stages of differentiation in the myeloid lineage: chronic myeloid leukemia (t9:22, Philadelphia chromosome; bcr-abl); acute promyeloid leukemia (t15:17, PML/retinoic acid receptor); acute myeloid leukemia (multiple possibilities including using both an activation signal (such as activation of the IL-3 receptor) and a block in apoptosis). CML is effectively treated by Gleevec, which specifically blocks the bcr-abl tyrosine kinase. APL is treated by retinoic acid, which reacts with the retinoic acid in the fusion product and allows the affected cells to differentiate. Treatment of AML by differentiation therapy is still in the experimental stage. From Sell (2005)

follicle gives rise to trichoepitheliomas, which vary in cellular differentiation but usually contain both keratitic and basal regions, as well as clear cells characteristic of hair follicle (Brown et al., 1998). Cells in the basal layer may give rise to basal cell carcinomas or squamous cell carcinomas. Overexpression of Ras in the more highly determined basal cells of the skin produces squamous cell carcinoma (Waikel et al., 2001; Arnold and Watt, 2001), and induced expression of the c-myc gene in the non-proliferative suprabasal cells reactivates the cell cycle and leads to hyperplasia (papillomas) (Pelengaris et al., 1999). Papillomas do not progress to invasive tumors (Pelengaris et al., 1999). Examination of the cell populations in skin cancer demonstrates that the malignant cells can also differentiate, but that the proliferative transit-amplifying cells of the cancer do not uniformly do so, unlike normal skin cells.

1.1.4 Malignant Cells May Become Benign

Pierce and Wallace (1971) published the results of an ingeniously simple experiment demonstrating that the proliferating cells in a squamous cell carcinoma of the skin can differentiate into non-proliferating terminally differentiated cells. They pulse-labeled proliferating squamous cell carcinoma cells with tritiated thymidine, which became incorporated into the newly formed DNA of cells while they were in the process of division. When the cancers were examined immediately after labeling, only the dividing cancer cells were labeled. However, when examined several days later, the terminally differentiated cells of the cancer and even the keratin produced by the cancer contained the label. The authors concluded that malignant cells can become benign. Thus, not all of the cancer transit-amplifying cells divide symmetrically, giving rise to two continually proliferating transit-amplifying cells; instead some cancer transit-amplifying cells divide asymmetrically, producing one daughter cell that remains a transit-amplifying cell and one that terminally differentiates and sometimes to two differentiating cells.

1.1.5 Comparison of Normal Tissue Renewal to Growth of Cancers

The difference between normal tissue renewal and cancer growth is that the number of cells that are produced by cell division in normal tissue essentially equals the number of cells that terminally differentiate in a given time period, so that the total number of cells remains constant. In contrast, in cancers, the proliferating transit-amplifying cells do not all terminally differentiate, and the number of cells in the cancer increases (Sell and Pierce, 1994). The stem cells in both normal tissue renewal and cancer growth consist of a small fraction of cells that are not actively proliferating, and that fraction serves as a reserve cell population. When a tissue stem cell divides, it gives rise to one daughter cell that remains a stem cell and one daughter cell that begins the process of differentiation by becoming a transit-amplifying cell (asymmetric division); thus, the stem cells remain in the tissue for long periods of time, essentially the lifetime of the

organism. The number of cells in a cancer increases with time, because the transit-amplifying cells give rise to two cells that do not mature and retain the potential to divide (symmetric division) or the mature cells do not die or both.

1.1.6 Normal Tissue Contains Stem Cells with Malignant Potential

Attempts to culture cells from normal tissues and cancers were well underway in the 1950 s (see below), and there were even some early studies suggesting that normal tissues contain stem cells with malignant potential. For example, in 1953, Harry Goldblatt, better known for his studies on renal hypertension (Goldblatt et al., 1934), found that malignant cells could be derived from normal rat myocardium (fibroblasts) if the cells were cultured for a long time in anaerobic conditions (Goldblatt and Cameron, 1954). Most normal tissue cells do not survive under these conditions. He and his co-worker suggested that normal tissue contains rare cells (stem cells?) with the potential for malignant change under selected culture conditions. On page 526 of their paper, they wrote, "It seems possible that in all embryonic, and even adult, normal tissues there may be scattered cells, or groups of cells of potentially neoplastic sort, which naturally possess the ability to use the fermentative, glycolytic mechanism, at least under anaerobic conditions, and that repeated, brief exposure of cultures of normal tissue containing such cells to an atmosphere deprived of oxygen, alternating with long periods when adequate oxygen is available, thus permitting recovery, might favor their multiplication and even interfere with the growth of the regional, normal cells." Given the previous observations of Otto Warburg and associates (Warburg et al., 1927) that a common feature between cancer cells and embryonic cells is the ability to use glycolytic metabolism, the implication is that normal tissues contain stem cells that retain the properties of embryonic stem cells, and that such cells can become malignant under selective growth conditions.

1.2 Theories of the Cellular Origin of Cancer

Two major theories of the origin of cancer have vied for acceptance in modern cellular biology: origin from stem cells and origin via de-differentiation of mature cells.

1.2.1 Embryonal Rest Theory of Cancer

The concept that cancers arise from stem cells in adult tissues was originally formulated as the embryonal rest theory of cancer. The first known theory of cancer proposed by Hippocrates, Celsus, and Galen was that cancer is caused by an excess of black bile, according to the humoral theory of disease (Shimkin, 1977). However, in the early and mid-1800 s, with the invention of the

microscope and an increasing capacity to interpret gross tissue changes, Joseph Claude Anselme Recamier (1829) and Robert Remak (1854) both reported that cancer tissue looked like embryonic tissue. These observations resulted in a new theory, namely, that cancer arises from embryonic cells that persist in the adult. This was formalized as the embryonal rest theory of cancer by F. Durante (1874) and Julius Cohnheim (Cohnheim and Congenitales, 1875). According to the embryonal rest theory, cancers grow out from small collections of embryonal cells that persist and do not differentiate into mature tissues in the adult. The embryonal theory of cancer thus served as a precursor to the stem cell theory of cancer as we know it today. However, during the last half of the 19th century, the embryonal rest theory of cancer fell out of favor, to be replaced by the de-differentiation theory of cancer (Sell, in press).

1.2.2 De-differentiation Theory of Cancer

In the latter half of the 19th century, there was an ongoing debate between those favoring the embryonal rest theory and those championing the de-differentiation theory of the origin of cancer (Bainbridge, 1914). The well-known surgeon and pathologist, Sir James Paget, wrote in 1853 that cancers came from morbid material in the blood, essentially a variation of the black bile theory (Paget, 1853). Even Rudolf Virchow, the "father of pathology," who described embryonic tissues in teratocarcinoma, concluded that cancers arose from connective tissue during chronic inflammation (Virchow, 1863). (Cohnheim was a student of Virchow.) The earlier observation by Sir Percival Pott, considered to be one of the first cancer epidemiologists, that chimney sweeps developed cancer of the scrotum (Pott, 1775; Potter, 1963) was interpreted to mean that cancers were due to de-differentiation of mature epithelial tissues induced by chemicals. Others proposed a "disequilibrium" between connective tissue and epithelium (Thiersch, 1865), a changed "habit of growth" of normal cells (Benecke, 1892 1893–; Adami, 1909), or the loss of "restraining influences" of the body on displaced tissue cells leading to de-differentiation of mature cells (Rippert, 1904). Cancer was believed by Amedee Borrel (1907) and a number of other scientists to be caused by infectious parasites. Later, Peyton Rous identified the Rous sarcoma virus as a cause of cancer in fowl (Rous, 1911). Subsequently, virus infections of stem cells, such as by human papilloma viruses, have been demonstrated to be a cause of some epithelial cancers. In squamous cell carcinoma of the uterine cervix associated with human papilloma virus infection, the initially infected and transformed cell is the basal stem cell, but productive infection requires keratinocyte differentiation (Munger and Howley, 2002; Garland, 2002). Finally, in 1914, sea urchin cells embryos were found by Theodore Boveri to have an abnormal chromosome composition, and it was concluded that genetic changes in mature tissue cells cause cancer via de-differentiation (Boveri, 1914). The cumulative impact of these observations lent little support to the embryonal rest theory of cancer. By 1914, William Seaman Bainbridge, in his authoritative book "The Cancer Problem"

(Bainbridge, 1914), concluded, "The congenital or embryonic theory of the origin of cancer has received no support whatever from the experimental and comparative investigations of recent times." In any case, physicians of that time were overburdened with treatment of infectious diseases, such as tuberculosis and syphilis, and there was no impetus to study cancer as there is today. De-differentiation remained the dominant theory of the origin of cancer into the 1980 s, despite observations on teratocarcinoma which proved that at least this cancer, if not others, arose from stem cells and contained stem cells.

1.3 Teratocarcinoma, Stem Cells, and Cancer

Teratocarcinoma is a malignant cancer that arises from the germinal stem cells, usually in the testes or the ovaries of a young adult (O'Hare, 1978). This cancer usually grows rapidly, but responds well to therapy, as exemplified by the case of Lance Armstrong. Studies beginning in the 1950 s on teratocarcinoma proved not only that these cancers arise from stem cells, but also, more generally, that cancers contain stem cells, and that therapy can be directed against these cancer stem cells.

1.3.1 Teratocarcinomas Contain Pluripotent Stem Cells

Barry Pierce and his colleagues (Pirece et al, 1978) demonstrated that teratocarcinomas contain the same population of cells as is present in normal tissue: stem cells, transit-amplifying cells, and differentiated cells. They demonstrated that over 99% of the cells of most teratocarcinomas belong to mature differentiated tissues, including derivative cells from all germ layers (skin, glands, connective tissue, vessels, neural tissue, bone marrow, etc.) as well as yolk sac and placental tissue (Pierce et al., 1978, 1960; Pierce and Dixon, 1959; Pierce and Spears, 1988). In fact, the production and secretion of alpha-fetoprotein (AFP) by the yolk sac component, and of chorionic gonadotropin (CGH) by the placental component of teratocarcinomas, indicate that the teratocarcinoma stem cells are totipotent, since they differentiate into cells both of the body of the embryo and of the yolk sac and placenta. AFP and CGH are considered to be oncodevelopmental markers; blood levels of these markers are used for the diagnosis and follow-up of treatment of teratocarcinomas (Chan and Sell, 2001). The only malignant cells in the teratocarcinoma are found in structures in the tumor that resemble early embryos (embryoid bodies). In vitro culture or transplantation of teratocarcinomas to syngeneic recipients can only be done using cells from the embryoid body (Pierce et al., 1960). Therefore, the embryoid body cells possessed two classical properties of cancer stem cells: the ability to grow in culture and the ability to initiate tumor growth upon transplantation. Pierce's group concluded that cancers are essentially caricatures of normal tissues (Pierce and Spears, 1988). They also

proposed that cancers can be treated by induction of differentiation of the cancer cells (Pierce and Spears, 1988). In fact, teratocarcinoma cells may be forced to differentiate when treated with retinoic acids, a principle that has been applied to other types of cancer (Sell, 2004a; Camacho, 2003). However, when treatment is discontinued, the cancer will regrow from those cancer stem cells that are resistant to differentiation therapy. Resistance to therapy is another classical property of cancer stem cells that will be illustrated below for leukemia.

1.3.2 Teratocarcinomas Arise from Stem Cells

Leroy Stevens (1964) demonstrated that teratocarcinomas arise from normal stem cells. Using a mouse strain in which teratocarcinomas arose spontaneously, Stevens transplanted normal testicular germ cells (Stevens, 1967), tubal mouse eggs (Stevens, 1968), or early mouse embryo cells (Stevens, 1970), from their natural sites to abnormal tissue sites. He found that, in these sites, teratocarcinomas appeared. Thus, apparently normal stem cells could be transformed into cancer cells if removed from their normal tissue environment and placed into an unnatural niche. A proposed lineage of germinal cell tumors according to the stage of maturation arrest of the transit-amplifying teratocarcinoma cells and based on the model of Pierce et al. (1978) is illustrated in Fig. 2B. Mintz and Ilmmensee (1975; Ilmmenesee, 1978) then demonstrated that transplantable teratocarcinoma stem cells can give rise to normal tissues in mosaic mice formed from injection of teratocarcinoma cells into the blastocyst of a normally developing embryo. Clearly, the malignant teratocarcinoma cells differentiated into normal benign cells of various tissues when placed in a controlling and nurturing environment. Richman Prehn (2005) proposed that stem cells in adult tissue are restricted through silencing of embryonic genes by the adult tissue microenvironment. He further postulated that when embryonic genes are silenced in tissue stem cells, mutations take place in those embryonic genes that go un-repaired. Then, an altered tissue environment allows activation of mutated embryonic genes in tissue stem cells that can lead to expression of a cancer phenotype.

1.3.3 What Have We Learned from Teratocarcinomas?

From the study of teratocarcinomas, we have learned that (1) cancers arise from maturation arrest of stem cells; (2) cancers contain the same cell populations as do normal tissues (stem cells, transit-amplifying cells, and terminally differentiated cells); (3) proteins released by the embryonic components of a teratocarcinoma can be used to detect the tumor; (4) malignant cells can become benign; (5) differentiation of cancer cells can be forced (differentiation therapy); (6) therapeutic regimens can be directed against the cancer transit-amplifying cells, but the cancer may regrow from the treatment-resistant cancer stem cells when treatment is discontinued. Clearly, these findings support a stem cell theory of cancer. However, until the 1980 s, many

investigators believed that teratocarcinomas were different from other cancers, and so still supported the de-differentiation theory of cancer. The paradigm for de-differentiation was the result of experimental models of chemical hepatocarcinogenesis. In these models, the cellular changes that were seen during induction of hepatocellular carcinoma (HCC) were held up as examples of de-differentiation. However, as we will see next, more recent examination of the cellular events during experimental chemical hepatocarcinogenesis actually supports the idea that the liver stem cell is the cellular origin of HCC. Given this change in paradigm during the last 30 years, we have seen a swing back to the stem cell theory of cancer reflected in a spate of reviews on cancer stem cells (for example, see Sell, 2004a, 2006a; Reya et al., 2001; Wicha et al., 2006), as well as books such as this one.

1.4 Chemical Hepatocarcinogenesis and the Stem Cell Origin of Cancer

Prior to the 1980 s, analysis of the experimental models of chemical hepatocarcinogenesis in the rat centered on the development of enzyme-altered foci and the so-called preneoplastic nodules and persistent nodules. These nodules appeared to depict a sequence of morphologic and histologic changes extending from the histologic structure of normal liver to that of hepatocellular carcinoma (HCC). Thus, it is not surprising that the nodules were considered to arise from altered mature hepatocytes and to represent precursor lesions to HCC (Farber, 1956; Bannasch, 1984; Aterman, 1992). The conclusion that these nodules were precursors to cancer implied that HCC arises by de-differentiation from mature hepatocytes.

1.4.1 Oval Cells

De-emphasized in the interpretation of the nodular changes preceding HCC was the fact that many of the hepatocarcinogenic regimens produced a notable proliferation of small oval cells (Farber, 1963). Later oval cells were shown to produce AFP, which suggested that these cells could represent fetal liver cells (Sell, 1989, 2001). Different kinetics of production of AFP associated with different preneoplastic changes suggested that multiple cell types in the liver lineage might be involved in the early carcinogenic process (Sell and Becker, 1978). In particular, during the cyclic feeding of acetylaminofluorene (AAF), a model designed to produce maximal preneoplastic nodule formation (Teebor and Becker, 1971), AFP elevations appeared early, before preneoplastic nodule formation; further AFP was not found in nodules (the presumed precursors to HCC), but rather in small oval cells located in-between the nodules (Sell, 1978). The fact that the HCC produced by this regimen also produced AFP was the first clue that HCC did not arise from nodular cells per se, but from putative

liver stem cells (oval cells; Sell et al., 1980; Sell and Leffert, 1982; Sell, 2008). As different models were examined for AFP elevations and early cellular changes, it became more obvious that different carcinogenic regimens produce different cellular changes, and that different cells in the liver lineage must give rise to the cancers, depending on the regimen (Sell, 2001). For a recent review on how the study of AFP production during normal development, after liver injury, and during chemical hepatocarcinogenesis led to the conclusion that liver cancer originates from stem cells, see Sell (2008).

1.4.2 Cellular Changes in the Liver Preceding Development of Liver Cancer

From among the many carcinogenic regimens that have been devised, four that produce different cellular changes during hepatocarcinogenesis will be adumbrated: diethylnitrosamine (DEN), furan, the Solt–Farber model, and choline deficiency combined with ethionine (CD-E) or AAF model. Interpretation of these models is quite complex and will only be summarized here. For a recent extensive review by the author citing many earlier references, see Sell (2006b). Briefly, DEN-induced HCCs appear to arise from hepatocytes; furan tumors arise from bile duct cells, Solt–Farber model HCCs arise from bipolar ductal cells, and CD HCCs arise from periductular oval (stem) cells.

Thus, these four different carcinogenic protocols result in distinctly different patterns of premalignant cell proliferation implicating four types of cells as possible precursors to cancer: hepatocytes (DEN), duct progenitor cells (furan), ductular bipolar precursor cells (Solt–Farber), and periductular stem cells (CD models). The "blastomas" of young animals provide the missing link between fetal hepatocytes and adult liver lineage cells at which stage of maturation arrest occurs in the formation of HCC (Fig. 2C; Kasai and Watanabe, 1970; Haas et al., 1989). In summary, reinterpretation of the cellular changes in the liver that are seen using various chemical hepatocarcinogenic regimens actually supports maturation arrest of the liver cell lineage and the stem cell theory of cancer. Most investigators now favor the stem cell origin of cancer over de-differentiation models, although with some reluctance. The two-step model of initiation and promotion of skin cancer provides the most convincing argument for the stem cell origin of at least this cancer (Rous and Kidd, 1942; Friedwald and Rous, 1944; Berenblum and Shubik, 1947; Berenblum, 1941, 1954; Boutwell, 1964).

1.5 Skin Cancers Arise in Self-Renewing Stem Cells (Initiation and Promotion)

The time elapsed between the application of a carcinogen to the skin (initiation) and the elicitation of cancer by promotion demonstrates that the skin cancers that result from this treatment arise from long-surviving stem cells. In the classic model, benz(*o*)pyrene, the initiator, is painted onto the skin. This chemical

binds to DNA in the skin cells, causing a permanent genetic alteration (initiation). However, cancers will not arise unless a proliferative stimulus is subsequently given (promotion). This stimulus is provided by treatment of the initiated skin site with phorbol ester. Thus, the initiation event induces genetic damage, and the promoter then stimulates the damaged cells to proliferate, leading to cancer. Initiation must occur before promotion. If promotion is performed prior to initiation, cancers will not develop. In the original experiments of Peyton Rous (Rous and Kidd, 1942; Friedwald and Rous, 1944), initiation was accomplished by painting the ear of a rabbit with coal tar. This produces genetic lesions in the epidermal cells through induction of DNA/carcinogen adducts that cannot be repaired. If no further insult occurs, tumors do not develop. However, if the site is wounded by scraping with a cork borer, epithelial cancer appears at the edge of the wound.

The time between initiation and promotion is the critical element that implicates the stem cell in the skin as the initiated cell. This interval can be days, or even months or years, in length (Berenblum and Shubik, 1947; Berenblum, 1941, 1954; Boutwell, 1964; Van Duuren et al., 1975). In order for tumors to grow in this model, the initiated cells must survive from the time of initiation to the time of promotion. Given the well-established fact that all cells in the skin, except for the self-renewing progenitor cells, turn over completely every 2–3 weeks in mice and about 1 month in humans (Potten and Morris, 1988; Watt, 1989), the only way in which the initiated cells could still be present, if months or years have passed since initiation, would be if initiation had occurred in the self-renewing progenitor cell population. In the course of the year or more between initiation and promotion, all of the transit-amplifying cells would have been replaced by newly generated cells from the basal stem cells. Thus, in the initiation–promotion model for skin carcinogenesis, the initiated cell must be a self-renewing progenitor cell.

2 Cancer Stem Cells

The premise in the preceding section of this chapter was that cancers arise from stem cells. Now we will discuss in more depth the evidence that cancers also contain stem cells. Within the last 10 years, there has been an extraordinary revival of the concept of cancer stem cells (Sell, 2004b, 2006a; Sell and Pierce, 1994; Reya et al., 2001; Sutherland et al., 1996; Bonnet and Dick, 1997). However, the biological properties of cancer stem cells have been studied for half a century.

2.1 Properties of Cancer Stem Cells

The critical properties of cancer stem cells are transplantability, the ability to grow in vitro, and the capacity to survive therapy directed against the cancer amplifying cells (Buick, 1980).

2.1.1 Transplantability

In 1952, Harry Green pointed out that embryonic and cancer tissues, but not normal or hyperplastic tissues, will grow in immune-privileged sites in "alien" animals (Green, 1952). Transplantable tumor cells, the so-called tumor-initiating cells, were found, through dilution studies, to comprise from 1 in 30 to 1 in 1,000 of the cells in solid cancers (Reinhard et al., 1952a; Hewitt, 1952). For example, to achieve a success rate of 50% in transplantation requires the injection of 300 adenocarcinoma cells (Reinhard et al., 1952b).

2.1.2 Growth In Vitro

The frequency of tumor-initiating cells was later found to be of the same order of magnitude as the frequency of cells that survive and grow in soft agar. In 1955, Puck and Marcus developed the soft agar culture method (Puck and Marcus, 1955). Using this approach, Salmon (1952) found that 1 in 1,000 to 1 in 100,000 cells would form colonies (called "tumor colony-forming units"). This range of proportions of tumor colony-forming units is similar to the proportions found more recently for leukemic tumor-initiating cells (Sutherland et al., 1996; Bonnet and Dick, 1997; Lapidot et al., 1994). The rediscovery of the properties of tumor growth in vitro and tumor initiation after transplantation has served as a stimulus for the present resurgence in interest in cancer stem cells.

2.1.3 Resistance to Therapy

The ability of a small population of tumor cells to resist radiation therapy was studied using a library of transplantable HCCs in rats developed by the late Harold Morris at Howard University (Morris and Meranze, 1974). Characterization of the cellular composition and biological behavior of Morris hepatomas in the 1960 s revealed that these cancers contained cells with stem cell-like characteristics including the ability to be cultured in vitro (Morris and Meranze, 1974). Transplantation of the Morris hepatomas revealed heterogeneity in growth properties. Some of the Morris hepatomas could be transplanted by means of just a few cells, which then grew very quickly, whereas others could only be transplanted by means of large numbers of cells and grew very slowly (Morris and Meranze, 1974; Looney et al., 1971).These findings demonstrated the first property of cancer stem cells noted above, i.e., transplantability. Finally, extensive studies on the response to irradiation treatment, by William Looney's group (Kovacs et al., 1977), demonstrated that after high-dose radiation, the tumors were able to regrow, indicating the presence of a therapy-resistant cancer stem cell. More recently, as an example of resistance to therapy, highly tumorigenic subpopulations of cancer-initiating cells derived from human glioblastomas were found to resist radiation because of their increased protection against DNA damage (Rich, 2007), thus supporting the idea

(presented above for leukemia) that cancer stem cells are resistant to standard therapies. Now we will address the exciting recent observations that suggest the potential for that cancer stem cells to be isolated by the use of stem cell markers, allowing investigators to study the properties of these isolated cancer stem cells.

2.2 Isolation of Cancer Stem Cells?

The recent interest in cancer stem cells derives from the possibility that cancer stem cells can be isolated by flow cytometry using stem cell markers, for example, breast (Al-Hajj et al., 2003; Shipitsin et al., 2007) and liver cancer (Yin et al., 2007; Ma et al., 2007, 2008; Fang et al., 2008; Yan et al., 2008; Zen et al., 2007; Yamashita et al., 2008; Kaposi-Novak et al., 2006) markers .

2.2.1 Breast Cancer

Human primary breast cancer cells can be fractionated by cell sorting into two major populations: CD44+CD24−ESA+ and CD44−CD24+ESA−. ESA stands for epithelial cell-specific antigen. Primary tumors show mixtures of these two cells types (Shipitsin et al., 2007) and there is a high degree of heterogeneity of expression of these markers by immunohistochemistry, indicating that tumors are composed of at least two types of cells (Liao et al., 2007). One of six transplantation takes was obtained by injection of 100 cells from a very highly purified CD44+CD24−ESA+ population (Al-Hajj et al., 2003). Thus, the frequency of tumor-initiating cells in the purified population was similar to that reported in 1952 for whole tumor cell populations by Reinhard et al. (1952b); this group obtained 6% takes with injection of 18 cells. A higher transplantation frequency could be obtained when CD24−CD44+ESA+^{low} cells were sorted from CD24−CD44+ESA+^{high}cells, but the number of transplant attempts was too low for significance to be attained (Al-Hajj et al., 2003). In any case, these results suggest that two types of breast cancer cells can be separated on the basis of these markers: a stem cell-like population and a transit-amplifying type cell population. After transplantation of the CD44+CD24−ESA+ stem cell-like breast cancer cells, the growing tumor reconstitutes both cell populations, suggesting that the tumor-initiating stem cell-like population could produce progeny expression markers of the non-tumor-initiating transit-amplifying like cell population (Al-Hajj et al., 2003). On the other hand, the properties of the non-stem population were not reported. Shipitsin et al. (2007) found that the CD44+ cells were more stem-like than were the CD24+ cells, in terms of molecular phenotype; they proposed that the CD24+ cells may "evolve" from the CD44+ population. This hypothesis presumes, of course, that both fractions are part of the epithelial cancer cell population. In our laboratory, when breast cancers from transgenic mouse models were examined, similar fractionations of breast cancer cells based

on expression of CD24 and CD44 also yielded two populations of cells: CD24+CD44− and CD24−CD44+ (Ma J, Guest I, Ilic Z, Grant D, Zang M, Glinsky G, Sell S, Fractionation of mouse mammary cancer stem cells, in preparation; see also Cho et al., 1998, for a similar model). The CD24+CD44− population is epithelial, whereas the CD24−CD44+ population is mesenchymal, suggesting that, in the mouse cancers, this type of fractionation actually separates epithelial cancer cells from fibroblastic stromal cells.

2.2.2 Liver Cancer

Specific markers that appear to identify stem-like cells in human HCCs include CD133 (Yin et al., 2007; Ma et al., 2007, 2008), CD90 (Fang et al., 2008; Yan et al., 2008), ABCG2 (Zen et al., 2007), EpCAM (epithelial cell adhesion molecule; Yamashita et al., 2008), and Met (Kaposi-Novak et al., 2006). CD133 and CD90 expression is associated with fetal liver cell marker expression, tumor initiation, culture in vitro, and chemoresistance (Yin et al., 2007; Ma et al., 2007, 2008), all properties attributed to cancer stem cells.

2.2.3 Cancer Stem Cell Isolation and Transplantability

Although it is implied by the authors that fractionation of the human breast cancer cells, liver cancer cells, and other cancers results in purification of cancer stem cells, this may not actually be the case. Thus, so far, the population of the breast cancer cells with tumor-initiating ability has been found to consist of a much larger population of the cells than is the case for other cancer stem cell models. Additional work is required to determine whether there really is a stem cell in breast or liver cancer that is different from most of the other cells in that cancer. Also, the question of the efficiency of transplantability of human cancer cells to immunodeficient mice, as compared to the efficiency of transplantation of mouse cancer cells to syngeneic mice (Kelly et al., 2007), must be addressed. A marked discrepancy, if demonstrated, could explain why one population of cells from the human mammary cancers is unable to initiate tumors on transplantation to SCID mice. The application of mammary gland stem cell markers, such as endoglin and prion protein, could perhaps better characterize the putative breast cancer stem cell (Liao et al., 2007).

2.3 Cancer Stem Cells or Resting Cancer Cells

Although the concept that cancers contain stem cells has recently been revisited (Reya et al., 2001; Wicha et al., 2006; Tan et al., 2006), it is a topic has been debated for some time. For example, in 1994, contrasting views were published by Trott (1994) and by Denekamp (1994). Trott (1994) reviewed the data that a

proportion of cells ranging from 0.1 to 100% of all cells from transplantable mouse tumors meet the criteria of a tumor stem cell, i.e., "regrowth of the tumour preceded by clonal expansion from a single cell with unlimited proliferative potential." He concluded that tumors contain the same populations of cells as are found in normal tissue, consistent with the proposal of Pierce et al. for teratocarcinoma (see below; Pierce et al, 1978). On the other hand, Denekamp (1994), considering the same evidence, deduced that the putative cancer stem cells are merely the least differentiated cells in the cancer population and appear functionally and kinetically different from the mass of tumor cells. She concluded that the cancer stem cell is not as clearly definable as the normal tissue stem cell. The debate has now been renewed (Adams and Strasser, 2008). For example, Kern and Shibata (2007), using a mathematical analysis, point out that tumor-initiating capacity could be a varying probabilistic potential for all tumor cells, rather than a quantal and deterministic feature of a minority of tumor cells. Identification of tumor-initiating cell populations through the use of marker phenotypes could preferentially enrich for cells able to transplant tumors, but even with the best purification systems, the so-called non-tumorigenic cell population will contain up to 3% of tumorigenic cells (Li et al., 2007). Because the flow-cytometric separations depend on cell surface markers that may change expression or be masked by cell surface carbohydrates, it is possible that the fractionation procedure itself actually changes the ability to detect the marker. In addition, when we restate results obtained for human leukemia, the significance of transplantation of human cancer cells into SCID mice as an indicator of a property of cancer stem cells has come into question (Kelly et al., 2007). In contrast to the finding that only 1 in 250,000 human leukemic cells is transplantable, essentially all of the cells of a mouse B-cell lymphoma will produce tumors when injected into non-irradiated congenic recipients (Kelly et al., 2007), a reiteration of a finding originally reported in 1937 (Furth and Kahn, 1937). The possibility is thus raised that the tissue microenvironment of a SCID mouse limits the ability of the human leukemic cells to form a tumor; thus, the low fraction of transplantable cells in human leukemia could be due to an incompatible microenvironment (Kelly et al., 2007).

The major question is whether cancer stem cells, or cancer cells in G_0, responsible for the regrowth of cancer after treatment (Salmon, 1952) can be isolated and purified. There are certainly some doubts about this. If they can, it is possible that new therapies can be directed to the some specific characteristics of the cancer stem cell (Sell, 2004b, 2006a, 2007a; Sell and Pierce, 1994; Reya et al., 2001; Hill and Perris, 2007).

2.4 *Differentiation Therapy*

The ability of retinoids to induce differentiation of teratocarcinoma cells, mentioned earlier, proves the principle that differentiation of cancer stem cells

is inducible. The basic concept of differentiation therapy is that specific identifiable cell signaling pathways maintain "stemness" in cancer stem cells. If the stemness signaling pathways that regulate cancer stem cells can be modified, then the cancer stem cells should progress, becoming cancer transit-amplifying cells. As cancer transit-amplifying cells, they would be susceptible to other forms of therapy (Kelly et al., 2007; Furth and Kahn, 1937; Sell, 2007a; Hill and Perris, 2007; Till and McCulloch, 1961; Becker et al., 1963; Till et al., 1964; Makino and Kano, 1955).

2.4.1 Leukemia

Differentiation therapy has been most successfully applied to human leukemia. Leukemia is a malignant cancer of the blood and lymphoid system. It is manifested by a massive increase in immature white blood cells in the circulation. The presence of so many white blood cells in the blood causes the color of the blood to change from red to creamy or white. The term leukemia means white blood (leukos – white; haima – blood). Because of the replacement of the normal immune and inflammatory cells with leukemic cells, patients with leukemia usually die, because they are unable to fight off infections. Leukemia is caused by a failure of the cells in the white blood cell lineage to mature into functional cells (maturation arrest).

The Leukemic Stem Cell

The first definitive demonstration of tissue-specific stem cells for the hematopoietic system was in 1961 (Till and McCulloch, 1961); small numbers of normal mouse bone marrow cells were transplanted by intravenous injection into heavily irradiated mice. After 10–14 days, the spleens of these recipient mice contained nodules of maturing and mature blood cells. Thus, stem cells of the bone marrow could give rise to hematopoietic colonies in this system in which each colony was shown to be a single clone (Becker et al., 1963; Till et al., 1964). Even before these elegant observations, Furth and Kahn in 1937 demonstrated that leukemia of mice could be transplanted via a single cell (Furth and Kahn, 1937), and Makino and Kano (1955) concluded that leukemia arises from a progenitor or stem cell. More recently, with the use of the nude immunodeficient mouse as a recipient for human cancer cells, an approach first reported in 1969 (Rygaard and Povlsen, 1969), the transplantability of human leukemic cells has been examined. Of special interest was the finding that only 1 in 250,000 or so leukemic cells is able to transfer acute myeloid leukemia (AML) to a SCID mouse (Sutherland et al., 1996; Bonnet and Dick, 1997; Lapidot et al., 1994). This observation indicates that leukemia cannot be transplanted by means of the tumor transit-amplifying cells, but only by means of the true tumor stem cells. When a pathologist looks at the cells in the blood or bone marrow of a patient with AML, essentially all of the leukemic cells appear to be blasts. This suggests that all of the AML cells are dividing transit-amplifying

cells. From that observation, we would predict that AML should be transplantable via any of the blast cells of the leukemia, but, in fact, the tumor transit-amplifying cells of human leukemia seem not to be able to initiate leukemia upon transplantation into SCID mice (Sutherland et al., 1996; Bonnet and Dick, 1997). Thus, tumor initiation is a property only of the leukemic stem cell. On the other hand, as recently discussed by Kelly et al. (2007), the transplantability of human leukemias in SCID mice may be limited by difficulties experienced by human cells in adapting to a foreign (mouse) milieu. In fact, Kelly et al. found, similar to Furth and Kahn (1937), that mouse AML can be transplanted to non-irradiated histocompatible recipients via any leukemic cell. Although there are still inconsistencies in the identification of leukemic stem cells, it is clear that leukemias arise from a block in the differentiation of cells in the myeloid pathway (maturation arrest; Sell, 2005).

Maturation Arrest Is the Critical Lesion in Leukemia

Myeloid leukemia is clinically classified on the basis of how rapidly the disease progresses, without treatment, into acute, subacute, or chronic, although there are many intermediate variations. Chronic myeloid leukemia (CML) is due to a maturation arrest at the myelocyte level; acute promyelocytic leukemia (APL) is due to an arrest at the promyelocyte level;, and acute myeloid leukemia (AML) is due to an arrest at the myeloid progenitor cell level. The stages of maturation arrest are directly related to gene rearrangements that result in constitutive activation of the cells (Sell, 2005).

An illustrative example is the gene translocations responsible for Burkitt's lymphoma, a B-cell tumor. The translocations leading to Burkett's lymphoma result in the insertion of an immunoglobulin promoter next to the c-myc gene (activates proliferation) and/or the Bcl2 gene (blocks apoptosis). Although the gene translocations occur in every cell of the body (including the hematopoietic stem cells and lymphocytic stem cell) in transgenic mice with these fusion genes, the molecular lesion is only manifested in cells that activate the Ig promoter, i.e., B cells (Cory et al., 1999; Park et al., 2005). Thus, the stage of maturation arrest is determined by the point of differentiation at which the promoter of the fusion transgene is activated.

Many translocations have now been identified in myeloid leukemia (Rowley, 1975). For the purposes of this discussion, only three situations will be presented here: (1) the t9:22 bcr-abl translocation (Philadelphia chromosome) in CML, which results in constitutive activation of tyrosine phosphorylase (Nowell, 1974); (2) the t15:17 PML/RARα translocation in APL; and (3) two of many possible translocations in AML: t12:13 (FLT3; IL-3R), which activates kinases, and 13q12 ITD FLT3, which blocks apoptosis. The stage of maturation arrest for each of these leukemias is determined by the level at which the transgene product acts (Fig. 2D). Specific differentiation therapy can be directed toward these lesions (Sell, 2005).

Leukemic Stem Cells and Response to Therapy

Cure of leukemia requires elimination of the most primitive leukemic stem cells, which carry the leukemic translocation or mutation, as well as the proliferating leukemic transit-amplifying cells (McCulloch, 2004). One of the properties of cancer stem cells is their resistance to chemo- and radiation therapy, as illustrated by the response of AML to cyclic chemotherapy. When AML is first detected, the tumor load is in the range of 10^{12} cells. In general, chemo- or radiation therapy will be effective in eliminating 99.9% of the AML cells (Baird, 2004). This kill percentage is consistent either with the idea that fewer than 1 in 1,000 of the AML cells is a stem cell that is resistant to the therapy or else with the idea that therapy is ineffective against a leukemic cell that is not dividing when the therapy is administered. Since chemo- and radiation therapies are directed against proliferating cells (the growth fraction) of the AML, the stem or resting tumor cell is not affected by the therapy. The frequency of the therapy-resistant cell is somewhat higher than the frequency of tumor-initiating stem cells that was determined via transplantation (Sutherland et al., 1996; Bonnet and Dick, 1997), suggesting that some of the chemo-resistant cells are not true leukemic stem cells (Kelly et al., 2007). In any case, when the therapy is discontinued in order to allow the normal hematopoietic cells to recover, the AML stem cells will also begin to regrow and will produce more leukemic transit-amplifying cells. Then, a second cycle of therapy will be given that will once again eliminate 99.9% of the leukemic cells. Since the putative AML stem cells will again be resistant, the tumor will again regrow. After four cycles of therapy, some leukemias will be cured, suggesting that, in some leukemias, the most primitive bone marrow stem cell is not mutated (Baird, 2004). On the other hand, the genetic change in many AMLs is present in the most primitive stem cells, which are resistant to chemotherapy. At this point, the curative therapy modality must be changed, to ablative irradiation or chemotherapy and bone marrow transplantation.

Differentiation Therapy for Leukemia

Effective differentiation therapy of leukemia involves blocking of the constitutive activation signal provided by the fusion products of the specific gene translocations (Sell, 2005). Such a block reverses the specific maturation arrest as shown in Fig. 2D.

Chronic Myeloid Leukemia

The active tyrosine kinase responsible for constitutive activation of myelocytes in CML can be effectively blocked by imatinib (Gleevec, manufactured by Novartis; formerly called ST1571) (Druker et al., 2001, 2006). Imatinib and related compounds (Puttini et al., 2006) block the binding site for ATP and ADP on the tyrosine kinase and prevent the kinase-mediated

phosphorylation of the substrate. Once this signaling pathway is blocked, the leukemic transit-amplifying cells are free to differentiate, and they eventually die by apoptosis.

Acute Promyelocytic Leukemia

Differentiation of APL cells is induced by treatment with retinoic acids, which react with the fusion gene product and cause its degradation (Melnick and Licht, 1999; Soignet et al., 1997). The fusion gene is the promyelocytic leukemia (PML) protein gene fused with the retinoic acid receptor gene. The fusion product (PML–RARα) inactivates the PML protein, which is required for normal formation of granules and for maturation of the promyelocyte. Reaction of retinoic acid with the fusion protein results in ubiquitinization and degradation of the protein. The maturation arrest is thereby removed, and the leukemic cells are free to differentiate (Melnick and Licht, 1999).

Acute Myeloid Leukemia

The situation for AML is much more complex than for CML or APL. The complexity arises because there is more than one molecular lesion in AML. The lesions fall into two functional classes: Class I, proliferative, and Class II, apoptosis inhibitory (Chalandon and Schwaller, 2005). Class II lesions by themselves cause myelodysplasia. The transition to AML occurs when both a Class I lesion and a Class II lesion are present, usually because a second mutation occurs in a cell already bearing a lesion. The combination of a proliferative lesion with loss of apoptosis is a double whammy, resulting in AML (Chalandon and Schwaller, 2005). Because there are at least two lesions in AML, specific differentiation therapy requires that both lesions be treated. Thus, so far, such therapy has met with limited success, since agents for only one of the two signals are available in most cases.

Leukemic Stem Cells and Differentiation Therapy

As in the case for chemotherapy and radiation therapy, when differentiation therapy is discontinued, the leukemia will re-form from resistant leukemic stem cells. Chemotherapy and radiotherapy act on the transit-amplifying cells of the tumor, but the tumor stem cells are resistant. Differentiation therapy removes the block to maturation and allows the cancer transit-amplifying cells to differentiate and die. Again, however, the tumor stem cells are not susceptible, so that when differentiation therapy is discontinued, the tumor will regrow form from the cancer stem cell (Sell, 2004a, 2006a; Reya et al., 2001). At the end of this chapter, specific approaches to inhibition of the cancer stem cells using differentiation therapy will be discussed (see also Sell, 2006a).

Lessons Learned from Leukemia

From leukemia we have learned that the genetic lesions of cancer occur in the stem cells, and that the expression of these lesions later in the lineage of the white blood cells determines the stage of differentiation of the leukemia. Chemo- and radiotherapies act on the leukemic transit-amplifying cells, but the cancer stem cells are resistant. Newly developed techniques of targeted interference with the proliferation signals, or use of apoptosis inhibitors responsible for the maturation arrest, can allow terminal differentiation of leukemic cells.

3 Cancer Stem Cell-Directed Therapy

Regardless of whether cancers are maintained by stem cells or by a population of cells that are in G_0 at the time of treatment, the properties of the treatment-resistant cells can be exploited, in efforts to eliminate the therapy-resistant cells. The rationale for this approach is presented in Fig. 3 (Sell, 2006a,c, 2007a).

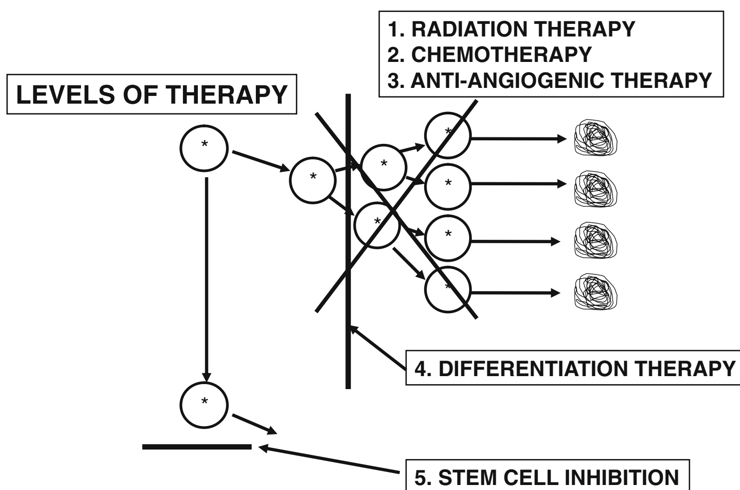

Fig. 3 Differentiation therapy of cancer stem cells. Chemotherapy, radiotherapy, and anti-angiogenic therapy are directed at the actively proliferating transit-amplifying cells of a cancer. When these therapies are discontinued, the cancer regrows from the therapy-resistant cancer stem cells. Differentiation therapy blocks the activation signals, causing maturation arrest. However, when differentiation therapy is discontinued, the cancer will re-form from the cancer progenitor cells. Stem cell inhibition is directed against the signals that keep a cancer stem cell a stem cell. By blocking or reversing the stemness signals, it may be possible to force the cancer stem cell to differentiate. From Sell (2006a)

3.1 Normal and Cancer Stem Cell Signals

A number of signaling pathways have been identified in normal (Ivanova et al., 2006) and cancer (Sell, 2007b; Dreesen and Brivanlou, 2007; Farnie and Clarke, 2007) stem cells. Signals that maintain normal stem cells are Wnt/β-catenin, Oct-4, Notch, BMP (bone morphogenic protein), JAK (Janus family kinase), and sHH (sonic hedgehog). Inhibition of these signals by RNA interference allows the stem cells to begin differentiation (Ivanova et al., 2006), establishing the principle that the stem cell properties of normal stem cells are maintained by stemness signals. A large number of signaling pathways have been identified in cancer cells (Sell, 2007b; Dreesen and Brivanlou, 2007; Farnie and Clarke, 2007). Most of them are shared by normal and cancer stem cells, including JAK-Stat, Notch, PI3K/AKT, NF-κB, Wnt, and TGF-β (Dreesen and Brivanlou, 2007). Although some of the signal proteins, such as Notch and NF-κB, may eventually be targeted (Farnie and Clarke, 2007; Zhou et al., 2007), it would appear more effective to target signals that at least are more prominent in cancer cells than in normal stem cells. Signals that have been specifically identified as being associated with cancer stem cells and poor prognosis are Bmi1, Oct-4 (Pou5F1), EED, and Lmo4 (Glinsky, 2006). Specific inhibitors need to be identified and ways found to deliver the inhibitor effectively in vivo.

3.2 Cancer Stem Cell Inhibition

Possible inhibitors of cancer stem cell signaling pathways include inhibitory RNA (iRNA) and specific molecular inhibitors of the signaling pathway (Sell 2006a, 2007b).

3.3 Inhibitory RNA

Potential targets for inhibition via iRNA are listed in Table 1. Whereas inhibition by iRNA has been shown in some instances in vitro, major problems exist in adapting iRNA inhibition for use in vivo (Pirollo and Chang, 2008; Pirollo

Table 1 Some potential targets for iRNA inhibition in cancer therapy

Cancer type	Targets
Breast	HER-2/neu, EGFR, epithelial specific antigen (ESA), CD44, Notch, TGF-β
Leukemia/non-Hodgkin's lymphoma Cancer stem cells	CD33, CD45, etc. Bmi1, c-kit, Notch 1, CD133, chemokine receptor CXCR4, CD34, SCA-1, Thy-1, EED, Oct-4, Lmo4
Solid tumors	SLAM family members CD48, CD150, and CD244

et al., 2007). First, in what form can the iRNA be injected, so that it is not degraded and has a chance to reach tumor cells intact? Possible forms include free, cyclodextrin polymer-conjugated, carbohydrate-modified, liposomal nanopeptide carrier, biologic nanopeptide vehicle (ENGeneICDeliver Vehicle), and lentivirus or adenovirus constructs. Obviously, such a long list implies that no single approach has worked well, and much work needs to be done to devise a successful and general mode of delivery. Also, the route of injection may be critical. For example, free iRNA would not be expected to survive to reach the cancer unless it is injected directly into the tumor. The various routes of delivery proposed, besides direct injection into the tumor, include systemic, nasal, intraperitoneal, and intrahepatic. Nanoimmunoliposome complexes of iRNA against HER-2 mRNA, encapsulated by a cationic liposome and decorated with anti-TfR single-chain antibody fragments, have been targeted at primary and metastatic lesions in SCID mice transplanted with human breast and pancreatic cancers (Pirollo et al., 2007).

3.4 Molecular Inhibitors

Molecular inhibitors for major stem cell signaling pathways are listed in Table 2, and the full names of these inhibitors appear in Table 3. Again, most of these agents have been shown to have some inhibitory effect on cancer cells in vitro, but how this information can be applied in vivo and whether or not the agents will have deleterious effects on normal tissue stem cells remain to be determined. Even if some of these approaches eventually do work out, a word of caution is needed because of the tendency of cancer stem cells to mutate and change characteristics, usually to a more malignant form (Nowell, 1976). This property makes the cancer stem cell a moving target for specific therapy.

Table 2 Some inhibitors of major stem cell signaling pathways

Signaling pathway	Inhibitor
JAK-STAT	APS
Notch	γ-secretase inhibitor (DAPT)
MAPK/ERK	RAF kinase inhibitors/U0126
PI3K/Akt	Rapamycin (LY294002)
NF-κB	I-κB, PTDC, Velcade
Wnt/β-catenin	NSAID, GSK-3, sFRPs, DKK, Axin
TGF-β (BMP)	SMAD6,7; Lefty1,2; Gremlin; SM16, etc.
Sonic hedgehog (sHH)	Cyclopamine
Oct-4/Sox2/Nanog	Tcf3

Adapted from Sell (2007a)

Table 3 Some molecular inhibitors of signaling pathways

Inhibitor	Full name of inhibitor
APS	Adaptor molecule (pleckstrin homology and SH-2 domains)
NSAID	Non-steroidal anti-inflammatory drugs
GSK-3	Glycogen synthesis kinase-3
sFRPs	Secreted Frizzled-related proteins
DKK	Dickkopf family (WIF-1, Cerebus)
DAPT	γ-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine t-butyl ester
SMAD6,7	Related to Drosophila Mad (Mothers against decapentaplegic), inhibitor of SMAD transcription factors for TGF-β pathway
Lefty1,2	Inhibitor of Activin activation of TGF-β pathway
Gremlin	Inhibitor of BMP activation of TGF-β pathway
LY294002	Selective PI3 kinase (PI3K) inhibitor
I-κB	Inhibitor of κB
PTDC	Sodium pyrrolidinethiocarbamate
U0126	MAP kinase inhibitor [1,4-diamino-2,3-dicyano-1,4-bis (*o*-aminophenylmercapto)butadiene ethanolate]
SM16	Small molecular inhibitor of TGF-β type I receptor kinase (ALK5)
Tcf3	Repressor of Wnt target genes
Velcade	Blocks NF-κB

From Sell (2007a)

4 Conclusions

And don't throw the past away
You might need it some other rainy day
Dreams can come true again
When everything old is new again

Peter Allen

What is old?

- Cancers arise from maturation arrest of stem cells.
- Cancers contain the same populations as do normal tissues: stem cells, transit-amplifying cells, and differentiated cells.
- Differentiation markers can be used for diagnosis, prognosis, and evaluation of treatment.
- The number of cancer stem cells in tumors varies enormously, from 1 in 1 to 1 in 10^6.
- Cancer stem cells can be grown in soft agar.
- Cancer stem cells initiate tumors upon transplantation.
- Cancer stem cells are resistant to chemotherapy and radiation.
- Cancer transit-amplifying cells can be treated by differentiation therapy.

What is new?

- Cancer stem cells can be identified via oncodevelopmental cell surface markers.

- Epithelial cancer stem cells can be isolated.
- Cancer signaling pathways can be identified.
- Selective agents that block cancer signaling pathways are being identified.
- Delivery mechanisms for blocking agents need to be developed.

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Molecular Regulation of the State of Embryonic Stem Cells

Yuko Fujiwara and Stuart H. Orkin

Abstract Pluripotency is a defining feature of embryonic stem cells (ESCs). A mechanistic understanding of pluripotency should shed light on fundamental aspects of development. In this chapter, we review the extrinsic factors, protein, and gene regulatory networks and epigenetics of ESCs. With the availability of human ESCs and the capacity to reprogram somatic cells to a pluripotent state, we hope that a comprehensive description of the control of pluripotency in ESCs will contribute to the use of these cells in regenerative medicine.

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1 Introduction

It has been nearly three decades since mouse ESCs (embryonic stem cells) were first isolated from the inner cell mass (ICM) of preimplantation blastocysts. The generation of hESCs (human embryonic stem cells) and recent success in reprogramming of adult somatic cells to hiPS (human-induced pluripotent stem cells) has brought extraordinary attention to the stem cell field. Mouse (m)ESCs have been used for gene knockout studies for about 20 years. In spite

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of the fact that mutant mice from mESCs have been extensively used, we still have an incomplete understanding of this unique cell type. ESCs proliferate continuously, yet unlike cancer or other transformed cells, they maintain pluripotency, the capacity to differentiate to any cell type. And unlike the ICM, which is a transient cell type and which differentiate to any cell lineages subsequently, ESCs proliferate without loss of pluripotency.

The study of ESCs began with culture of teratocarcinomas and embryonal carcinoma (EC) cells. Male mice of the 129 strain were observed to have a high incidence of spontaneous teratoma formation in the testes (Stevens and Little, 1954). These germ cell tumors contain randomly organized tissues containing cells of ectodermal, mesodermal, and endodermal origins. A subset of tumors was malignant (teratocarcinoma) and could be re-transplanted to immunodeficient recipients. Teratocarcinomas contain relatively undifferentiated cells known as ECCs (embryonal carcinoma cells) (Kleinsmith and Pierce, 1964). These ECCs can be expanded on mitotically inactivated MEFs (mouse embryonic fibroblasts), which provide necessary nutrients or trophic factors. ECCs are transferable to a new host and reform complex teratomas that are transplantable to another host. Since ECCs, as cancer-derived cells, are aneuploid, proper differentiation is not to be expected. Nonetheless, characterization of ECCs has played an important role in defining the normal embryonic counterpart. Such efforts culminated in isolation of pluripotent ESCs from the ICM. mESCs were first derived in 1981 (Evans and Kaufman, 1981; Martin, 1981). hESCs were not reported until 1998 (Thomson et al., 1998). ESCs have two distinguishing features: pluripotency and self-renewal. Unlike differentiated cells and/or progenitors, ESCs proliferate without loss of pluripotency under optimal culture conditions. As a reflection of pluripotency, ESCs contribute to all cell types, including germ cell lineage of the resulting chimeras, upon injection into mouse blastocysts. In contrast to ECCs, chimeras with ESCs do not develop tumors (Papaioannou et al., 1975). ESCs also can form teratomas upon subcutaneous or intramuscular injection.

A particularly convenient and useful property of ESCs is their capacity for in vitro differentiation (Keller, 2005). ESCs can be differentiated spontaneously or under supplementation with growth factors. In addition to differentiation to all embryonic lineages, recent studies have demonstrated that ESCs may differentiate into extra-embryonic lineages, such as trophoectoderm and extra-embryonic endoderm. Trophoblast separates at an early stage from the ICM under the guidance of transcription factors Cdx2 (caudallo-type homeobox transcription factor) and Eomes (Eomesodermin) (Niwa et al., 2005). Later, the hypoblast (extra-embryonic endoderm) separates from the maturing ICM controlled in large part by Gata6 (Fujikura et al., 2002). Forced expression of Gata6 in ESCs drives differentiation toward hypoblast-like cell, mimicking the in vivo situation. On the other hand, forced expression of Cdx2 drives ESCs to a trophoblast-like fate. Oct4 and Sox2, two of the central pluripotency factors, antagonize Cdx2 to prevent trophoectoderm commitment. Reduction of Oct4 expression below 50% of normal levels induces differentiation of ESCs into

trophectoderm accompanied by upregulation of Cdx2 and Eomes. However, overexpression of Oct4 promotes hypoblast differentiation from ESCs (Niwa et al., 2000). Additionally, Nanog antagonizes Gata6 to prevent differentiation toward mature hypoblast (Chazaud et al., 2006). Thus, the state of ESCs is under tight regulation by multiple factors acting either together or in antagonism. In this chapter we address how pluripotency of ESCs is maintained.

2 Extrinsic Factors

Mitotically inactivated MEFs sustain the ESC state. Numerous studies have sought to identify factors secreted from MEFs. One of the critical factors for mESCs is LIF (Leukemia inhibitory factor), a member of the IL-6 (interleukin-6) cytokine family. LIF, first described as an inducer of differentiation of M1 myeloid leukemia cells, supports survival and proliferation of mESCs without inducing differentiation (Smith et al., 1988; Williams et al., 1988). mESCs express the LIF receptor (LIFR), which consists of the LIF-specific receptor subunit LIFRβ and the common signal transducer gp130 (glycoprotein-130) (Yoshida et al., 1994). Binding of LIF to LIFR activates JAK (Janus-associate tyrosine kinase) which then phosphorylates gp130, establishing a docking site for proteins bearing SH2 (Src homology 2) domains, including the STAT (signal transducer and activator of transcription) family of transcription factors. STAT proteins are a family of transcription factors that are normally inactive within the cytoplasm and accumulate in the nucleus upon growth factor stimulation. Several reports indicate that Stat3 is a crucial downstream transcription factor of the LIF/gp130 pathway. Similar to other STAT family proteins, STAT3 activation is transient in normal cells. However, STAT3 is persistently activated in many tumors as a consequence of aberrant growth factor or tyrosine kinase signaling. In mESCs, the binding of LIF to LIFR triggers STAT3 phosphorylation by JAK. Thereafter, phosphorylated STAT3 forms homodimers that translocate to the nucleus to promote gene activation. Experiments validate STAT3's intimate involvement in LIF signaling. Forced expression of dominant-negative STAT3 causes spontaneous differentiation of ES cells, even in the presence of LIF (Niwa et al., 1998). ESCs expressing a fusion molecule consisting of STAT3 and the estrogen receptor ligand-binding domain can be maintained in a pluripotent state in the presence of estrogen without LIF (Matsuda et al., 1999). The basic helix-loop-helix transcription factor c-myc, an established accelerator of the cell cycle, appears to lie downstream of activated STAT3. By ChIP (chromatin immunoprecipitation) analysis, STAT3 has been detected at the promoter of c-myc in mESCs. LIF withdrawal is associated with decreased DNA-binding activity of STAT3 and concomitant downregulation of c-myc expression (Cartwright et al., 2005). Overexpression of a dominant-active form of c-myc is sufficient to maintain self-renewal of ESCs independent of LIF and bypasses the dominant-negative effect of STAT3. Expression of a dominant-negative form of c-myc antagonizes

self-renewal and promotes differentiation. These data strongly suggest that the LIF/STAT3 pathway is involved in self-renewal of mESC through c-myc activation. The human homologue of LIF was isolated in 1988 (Gough et al., 1988). Surprisingly human LIF is not essential for maintaining the state of hESCs (Daheron et al., 2004; Sato et al., 2004).

LIFR/gp130 signals also activate both MAPK (mitogen-activated protein kinase) and ERK (extracellular receptor kinase) pathways. ERKs phosphorylate cytoplasmic proteins, which shuttle to the nucleus where they modulate the activities of transcriptional regulators. Interestingly, the MAPK/ERK pathway is a pro-differentiation signal rather than contributing to self-renewal in mESCs. The addition of MEK inhibitors to the culture medium promotes self-renewal by preventing activation of a pro-differentiation pathway leading to enhancement of LIFR/STAT3 signals (Burdon et al., 1999). The mESC state reflects an overall balance between signaling of STAT3 for self-renewal and MAPK/ERK for pro-differentiation. In contrast, MEK/ERK are targets of fibroblast growth factor (FGF) pathway in hESCs. MEK/ERK activity is required to maintain pluripotency in hESCs (Li et al., 2007c).

While LIF/gp130/STAT3 signaling appears critical for self-renewal and pluripotency in mESCs, genes in the pathway are dispensable for pluripotency of ICM by gene knockout studies. Mutant embryos lacking any of these genes form normal ICM. LIF-deficient mice develop normally (Stewart et al., 1992), while LIF receptor-deficient mice show perinatal lethality (Li et al., 1995; Ware et al., 1995). Embryos deficient in gp130 die progressively after 12.5 dpc (Yoshida et al., 1996). Stat3-deficient embryos die around 6.5 dpc (Takeda et al., 1997). These findings in the early embryo and mESCs suggest that the LIF/gp130/STAT3 pathway is not the sole pathway for maintaining pluripotency.

BMP4 (bone morphogenetic protein 4) is another extrinsic factor that supports mESCs pluripotency and self-renewal. BMP4 acts as an anti-neurogenesis factor and as a mesoderm inducer in early mouse embryos (Hollnagel et al., 1999). However, BMP4 seems to have different effects on mESCs in collaboration with LIF pathway (Qi et al., 2004; Ying et al., 2003). BMPs initiate signaling from the cell surface by interacting with heterodimers of type I and type II serine-threonine receptors (Chen et al., 2004). Following binding, cytoplasmic proteins called R-Smads (receptor regulated - similar to mothers against decapentaplegic homologues) are activated by phosphorylation and form heterodimers with the common mediator Smad (Co-Smad, Smad4). These heterodimers translocate to the nucleus where they either inhibit or activate target genes. In the presence of LIF, BMP4 maintains ES cell pluripotency by activating Smad4, which then activates members of the Id (inhibitor of differentiation) gene family to suppress neural differentiation. The major effect of BMP4 on the self-renewal of mESCs is to antagonize both MAPK (mitogen-activated protein kinase) and ERK (extracellular signal-regulated kinase) signaling, which serves as pro-differentiation pathways. Inhibitor of ERK and MAPK pathways mimic the effect of BMP4 on mESCs. Knockout of Alk3 (activin receptor-line kinase 3), one of BMP4 receptors, impairs derivation of

mESCs from the ICM, a phenotype that is rescued by an inhibitor of MAPK (Beppu et al., 2000; Mishina et al., 1995; Qi et al., 2004). Interestingly, in the absence of LIF, BMP4 counteracts the LIF pathway, interacting with different R-Smad transcription factors (e.g., SMAD1, 5, and 8) that exert an inhibitory effect on Id genes and lead ES cells into non-neural fates, such as mesoderm and hematopoietic cells. The LIF and BMP pathways play bidirectional roles in maintaining the ESC state. Acting through the SMAD pathway, BMP inhibits neuroectoderm differentiation of ES cells, whereas LIF activation of the STAT3 pathway blocks BMP-induced endoderm and mesoderm. LIF activates pro-differentiation pathways (e.g., MAPK/ERK gene activity), while BMP inhibits this pro-differentiation pathway to maintain self-renewal. Therefore, a balance may exist between the effectors, STAT3, Smad, and ERK activity on stimulation of the respective receptors.

The behavior of mouse and human ESCs differs, particularly in their requirements for growth factors. As given above, LIF is insufficient to maintain hESCs (Daheron et al., 2004; Sato et al., 2004). The delicate balance and cooperation between LIF and BMP pathway is not evident in hESCs. Unlike mESCs, BMPs cause rapid differentiation of hESCs (Xu et al., 2002). High concentration of bFGF (basic fibroblast growth factor) (100 ng/mL) is routinely used to maintain hESC in culture. However, the mechanism of bFGF function in hESCs has not yet been elucidated. One of the effects may be related to the BMP signaling pathway (Xu et al., 2005). Under moderate concentrations of bFGF, Noggin, an inhibitor of BMP signaling activity, has a synergistic effect to maintain hESCs better than bFGF alone. It was shown in another cell culture system that bFGF interrupted BMP signaling by preventing the nuclear translocation of phosphorylated Smad1 (Pera et al., 2003) or by repressing Smad1 activity in the nucleus (Nakayama et al., 2003). Although bFGF does not inhibit Smad1 phosphorylation, bFGF may inhibit BMP/Smad pathway in hESCs. On the other hand, under high concentrations of bFGF, Noggin's effect is no longer apparent. Moreover, suppression of BMP activity by inhibitors is insufficient to maintain hESCs. This observation suggests that bFGF not only affects BMP pathway but also is involved in other signaling pathways.

Recent microarray gene expression profile data demonstrate the upregulation of TGFβ1/activin and gremlin 1 (a BMP antagonist), as well as the downregulation of Bmp4 by bFGF (Greber et al., 2007a). The roles of TGFβ/activin/nodal and FGF signaling pathways have been further characterized in hESCs (James et al., 2005; Vallier et al., 2005). Both activin and TGFβ have strong positive effects in hESCs in the presence of modest concentration of bFGF. TGFβ/activin/nodal are known to activate Smad2/3 (Valdimarsdottir and Mummery, 2005). Activation of Smad2/3 signaling is required for the maintenance of the undifferentiated state of ESCs (James et al., 2005). Recent reports suggest that supplementation of culture medium with Activin A is sufficient to promote self-renewal of hESCs (Beattie et al., 2005; Xiao et al., 2006). Nodal have been shown to inhibit neural differentiation in hESCs (Smith et al., 2008). TGFβ1 has been suggested to prevent hESC differentiation along

the primitive endoderm lineage (Poon et al., 2006). These data suggest that TGFβ/activin/nodal supports the undifferentiated state of hESCs with bFGF by preventing differentiation.

The canonical wnt/β-catenin pathway supports self-renewal of both mESCs and hESCs (Hao et al., 2006; Ogawa et al., 2006; Singla et al., 2006). Wnt signaling is endogenously activated in ESCs and is downregulated upon differentiation. WNTs are secreted glycoproteins that have widespread roles in tissue differentiation and organogenesis. The Wnt pathway consists of two distinct components. In the canonical WNT pathway, binding of WNT to a Frizzled/LRP-5/6 receptor complex leads to a signaling cascade responsible for the accumulation and nuclear translocation of β-catenin, which regulates transcription in the nucleus. Activation of the Wnt pathway with wnt3a-conditioned media (but not with purified wnt3a protein) supports the undifferentiated phenotype in ESCs and sustains expression of the ESCs-specific markers, such as Oct4, Nanog, REX1 in the absence of LIF (Sato et al., 2004). BIO, a chemical inhibitor of GSK3 (glycogen synthase kinase 3) has a similar effect on hESCs. GSK3 phosphorylates β-catenin, which then becomes a target for ubiqitination and subsequent proteosomal degradation. In mESCs, GSK3 activation/inactivation is closely controlled by the LIF/gp130 pathway. GSK3 is suppressed by PI3K (phosphoinositide-3 kinase)-Akt through the LIF/gp130 pathway; on the other hand, GSK3 is rapidly activated upon withdrawal of LIF. PI3K also activates Akt, which encodes a serine/threonine protein kinase. A constitutively active form of Akt (myr-Akt) mutant maintains the undifferentiated phenotypes in mESCs in the absence of LIF (Watanabe et al., 2006). Wnt signaling upregulates STAT3 expression, suggesting that the Wnt/Frizzle pathway balances synergistic effects with LIF/STAT3 pathway in mESCs (Hao et al., 2006). As such, it may constitute the pathway that substitutes for the LIF pathway in hESCs. Furthermore, the canonical Wnt signaling pathway has been shown to elevate the level of c-myc, a target gene of STAT3. Interestingly, GSK3 phosphorylates c-myc (Sato et al., 2004) to promote its degradation. GSK3 inhibited by BIO may prevent c-myc degradation by also blocking c-myc phosphorylation by GSK3. Thus, these Wnt and LIF pathways may converge on c-myc through STA3 and GSK3. However, recent report suggests a different probability. The cooperative interactions of the canonical Wnt/β-catenin, Activin/Nodal, and BMP signaling pathways for hESCs define the differentiation toward mesoendoderm/endoderm instead of supporting self-renewal and pluripotency (Sumi et al., 2008). Interestingly, in this chapter, the GSK3 inhibitor BIO supported self-renewal only at low concentrations, whereas BIO prominently induces nuclear translocation of β-catenin and mesoderm differentiation in hESCs at high concentrations. The authors observed the same effect with overexpression of catenin/ER (estrogen receptor) fusion protein by various 4OHT (4-hydroxy-tamoxifen) concentrations. At lower concentrations, which anticipate modest activation of β-catenin, hESCs were seemingly maintained in a self-renewal state, despite weak induction of mesoderm markers, whereas at higher concentrations of 4OHT undifferentiated hESCs were abolished. This

suggests that the canonical wnt/β-catenin pathway in hESCs has a biphasic role in controlling self-renewal and differentiation dependent on a specific threshold of β-catenin activity.

Although other growth factors have been reported to have a positive effect on hESCs including IGF-1, heregulin (Wang et al., 2007a), pleotrophin (Soh et al., 2007), shingosin-1-phosphate (S1P) (Avery et al., 2008), PDGF (Pebay et al., 2005), and neurotrophin (Pyle et al., 2006), the pathways involved have not been fully explained.

3 "Core" Transcription Factors

Studies of the past few years have revealed the central role of transcription factor networks in the maintenance of ESCs pluripotency and self-renewal. Recently Yamanaka's group demonstrated that the stem cell state can be imposed on somatic cells by forced expression of four transcription factors (Oct4, Sox2, Klf4, and c-myc) (Takahashi and Yamanaka, 2006). Many other groups followed this finding (Yamanaka, 2008). Surprisingly, the same set of transcription factors reprogram both mouse and human somatic cells, despite differences in growth factor requirements of their respective ESCs (Park et al., 2008a,b; Takahashi et al., 2007a,b). iPS cells (induced pluripotent stem cell) demonstrate that transcription factors indeed rule pluripotency. Oct4, Sox2, and Nanog have been considered "core" transcription factors for pluripotency and self-renewal of ESCs. First we review these "core" factors and then describe the extended network surrounding the "core" factors and their relation with the extrinsic network.

Oct4 was first identified as a gene exclusively expressed in pluripotent and totipotent lineages (Okamoto et al., 1990; Scholer et al., 1989). Oct4 is a transcription factor belonging to the POU (Pit-Oct-Unc) family that regulates the expression of target genes by binding to the octamer motif ATGCAAAT within their promoter or enhancer regions (Herr and Cleary, 1995). Oocyte contains Oct4 maternal transcript and protein until fertilization (Pesce et al., 1998). Significant, but relatively low, levels of OCT4 protein are also found in all cytoplasm of 2- and 4-cell embryos. Zygotic expression of Oct4 is activated from the 4-cell stage with the strong nuclear localization in all blastomeres throughout the morula stage (Palmieri et al., 1994; Scholer et al., 1990). Oct4 expression is restricted to the ICM at the blastocyst stage and later on to the germ cell lineage (Pesce and Scholer, 2000). Oct4 is also expressed in germ cell-related tumors (Cheng et al., 2007). Recently, reports described Oct4 expression in adult stem cells. However, this finding may be confounded by the existence of Oct4 pseudogenes (Liedtke et al., 2007). Because of its unique restricted expression pattern and chromosome localization within t-complex on chromosome 17 (Yeom et al., 1991), Oct4 was a candidate of some of embryonic t-lethal mutations. As expected, Oct4-deficient embryos have an early embryonic lethal

phenotype. Mutant embryos develop through cleavage and compaction to form blastocyst-like structures. Normal numbers of cells seem to be distributed to the prospective ICM region. These ICM-like cells are viable, however, they are unable to advance further to form primitive ectoderm and extra-embryonic endoderm. On the other hand, trophoblast lineages appear normal. Only trophoblast giant cells grow out from Oct4-deficient blastocysts in vitro. These data indicate that OCT4 is a crucial factor for generation of hypoblast and epiblast and maintenance of the pluripotent state during embryo development (Nichols et al., 1998). No clear functions for Oct4 have been identified in adult somatic stem cells (Lengner et al., 2007). Both hESCs and mESCs contain abundant OCT4 protein in the nucleus. Expression of Oct4 declines upon differentiation. Inactivation of Oct4 in embryo and ESCs causes spontaneous differentiation to trophoblast lineage (Niwa et al., 2000). However, constitutive Oct4 expression in mESCs is insufficient to maintain self-renewal without LIF (Niwa et al., 2000). Overexpression of Oct4 yields the same phenotype as STAT3 deficiency. This suggests that LIF does not regulate Oct4, and Oct4 does not regulate the LIF/STAT3 pathway. The Oct4 pathway appears to be a parallel pathway for maintaining ESCs self-renewal. Many Oct4 target genes also contain STAT-binding sites, suggesting that the two transcription factors may cooperate in ESCs (Tanaka et al., 2002).

Sox2 is a member of the SOX (SRY-related HMG box) DNA-binding protein family. POU and SOX proteins function together to regulate gene expression both positively and negatively (Remenyi et al., 2004). Several reports suggest cooperative activity between Oct4 and Sox2 on ESCs-specific enhancers, such as those at the Utf1, Fgf4, Lefty1, and Nanog genes (Kuroda et al., 2005; Nakatake et al., 2006; Nishimoto et al., 2001; Rodda et al., 2005; Tokuzawa et al., 2003; Yuan et al., 1995). Furthermore, Oct-Sox enhancers are important for the expression of Oct4 and Sox2 themselves, suggesting that these two transcription factors are regulated by a positive-feedback loop (Chew et al., 2005; Okumura-Nakanishi et al., 2005; Tomioka et al., 2002). Expression of Sox2 in early embryos parallels that of Oct4, as it is expressed in the ICM, and then in the early primitive ectoderm (epiblast), and germ cells (Avilion et al., 2003). However, unlike Oct4, Sox2 is also expressed in neural stem cells (Uwanogho et al., 1995; Zappone et al., 2000). Indeed, loss of Sox2 in the central nervous system yields a phenotype that is independent of Oct4 (Avilion et al., 2003; Miyagi et al., 2008). Both Sox2-deficient and Oct4-deficient embryos arrest at similar stages (Avilion et al., 2003). Blastocyst-like structures are formed in Sox2 mutants, but primitive ectoderm development is defective. The primary defect lies in the epiblast, as illustrated by chimera rescue experiments, in which wild-type ESCs were injected into Sox2-deficient blastocyst. In many 7.5 dpc chimeras, the entire embryo is derived from the wild-type ESCs, revealing the defect to be cell-autonomous to the epiblast. Consistent with this finding, no outgrowth from blastocysts occurs in culture. ICM isolated from Sox2-deficient embryos gives raise to trophoblast giant cells in culture. Silencing of Sox2 by RNAi (RNA interference) in ESCs induces differentiation into multiple lineages,

including trophoblast (Greber et al., 2007b; Ivanova et al., 2006). This result is consistent with a role of Sox2 in maintaining pluripotency, similar to that of Oct4. SOX2 protein shuttles between the cytoplasm and nucleus during early embryogenesis (Li et al., 2007b). SOX2 protein is detected in the cytoplasm of growing oocytes. In contrast to OCT4 protein, maternal SOX2 actively enters the nucleus by the 2-cell stage. However, SOX2 is exclusively cytoplasmic in trophoblast at the blastocyst stage. Unknown mechanism for active protein export out of nucleus in trophoblast has not yet been elucidated (Avilion et al., 2003). These events occur independent of zygotic Sox2 transcription, which begins in the late morula, as maternal SOX2 protein in the cytoplasm of trophoblast is distributed identically in wild-type and Sox2 null blastocysts (Avilion et al., 2003). Recently, a nuclear translocation signal has been identified within the HMG box of several SOX proteins. SOX10 requires active nucleo-cytoplasmic shuttling for transactivation of target genes in vitro (Rehberg et al., 2002), whereas sex reversal can be induced in cultured XX gonads using an inhibitor that results in nuclear sequestration of SOX9 (Babaie et al., 2007; Gasca et al., 2002). SOX2 has two distinct nuclear translocation signals. The Dmu-mSox2 mutant gene has mutations in these signals and fails to remain in the nucleus, but yet is competent to interact with wild-type SOX2 (Li et al., 2007b). Dmu-mSox2 is unable to cooperate with OCT4 at Oct-Sox target promoters in ESCs. Since Dmu-mSox2 can still interact with wild-type SOX2, it inhibits the activity of wild-type SOX2 in a dominant-negative fashion, and subsequently suppresses the activity of downstream genes, such as Oct4 and Nanog. Overexpression of Dmu-mSox2 in ESCs triggers progressive doublings of cell ploidy (<8 N), accompanying trophoblast differentiation. These results resemble the knockout of Sox2. In toto, these data indicate that SOX2 maintains stem cell pluripotency by shuttling between the nucleus and cytoplasm and in cooperation with OCT4 prevents trophoblast differentiation and polyploid formation in ESCs. Surprisingly, overexpression of Oct4 restores self-renewal in Sox2 null cells (Masui et al., 2007). Sox2-null-Oct4-rescued cells seem normal and LIF-dependent for proliferation. Stem cell markers (Fgf4, Nanog, Utf,1 and Foxd3) are expressed at relatively high levels in the rescued cells. These observations suggest that Sox2 may be dispensable at a subset of Oct-Sox enhancers, or other Sox proteins, such as Sox15, may compensate (Maruyama et al., 2005) with overexpressed Oct4.

The third "core" factor, Nanog, a member of NK homeodomain transcription factor family, was first identified by Chambers et al. and Mitui et al. using different strategies (Chambers et al., 2003; Mitsui et al., 2003). The name "Nanog" derives from "Tír na nÓg", the mythological Celtic land of the "ever young". Nanog mRNA is first detected in the interior cells of the compacted morula and then restricted to the ICM. In later blastocysts, Nanog expression is further restricted and excluded from the primitive ectoderm. Nanog is expressed in germ cells during embryogenesis, but downregulated thereafter. Adult tissues do not express Nanog. Upon differentiation of pluripotent cell lines, such as ESCs, EGCs, and ECCs, Nanog expression is progressively extinguished. Unlike Oct4 and Sox2, Nanog is not a maternal factor. Mouse embryos lacking

Nanog fail to develop due to the absence of primitive ectoderm (Chambers et al., 2003; Mitsui et al., 2003). A clear epiblast and extra-embryonic ectoderm at 5.5 dpc do not form. Nanog-deficient blastocysts are indistinguishable from wild-type embryos. The ICM fails to proliferate, and yet differentiates to parietal-endoderm-like cells. These data suggest that Nanog expression is indispensable for the maintenance of primitive ectoderm in the embryos and prevents differentiation into extra-embryonic endoderm. Consistent with these findings, Nanog represses Gata6 to prevent ICM differentiation to hypoblast (Mitsui et al., 2003; Singh et al., 2007). mESCs cannot be derived from Nanog-null blastocysts (Mitsui et al., 2003).

In contrast with these in vivo results in Nanog-deficient embryos, Nanog-null ESCs can be generated under specific circumstances. mESCs with a conditionally modified Nanog locus were manipulated to form Nanog-null ESCs. Surprisingly, ESCs lacking both Nanog alleles may remain undifferentiated, yet are prone to differentiate (Chambers et al., 2007). Nanog-deficient mESCs express stem cell markers (Oct4, Sox2). Thus, Nanog is required for the establishment of ESCs, but dispensable for their maintenance (Chambers et al., 2007; Mitsui et al., 2003). Nanog expression is mosaic within ESCs population and appears to undergo oscillary circuits. This dynamic expression pattern is compatible with the existence of Nanog-deficient ESCs (Singh et al., 2007). Like Nanog-deficient ESCs, the Nanog negative population is susceptible to differentiation signals (Chambers et al., 2007). In contrast, overexpression of Nanog sustains mESCs pluripotency in the absence of LIF and or STAT3 activation. Thus, the Nanog and STAT3 pathways are independent (Chambers et al., 2003). However, ChIP (Chromatin immunoprecipitation) analysis suggests that Nanog may be a direct downstream effector of the LIF/STAT3 pathway in maintaining pluripotency (Suzuki et al., 2006). Further work is needed to confirm the direct link between Nanog and the LIF/STAT3 pathway. Recent reports suggest that NANOG is a direct target of TGFβ/activin-mediated SMAD signaling in hESCs (Xu et al., 2008).

The three “core” factors are integrated with LIF/BMP/Wnt pathways, cooperate with each other to prevent differentiation, and maintain pluripotency by a delicate balance. These “core” factors do not act in isolation, but rather within a network comprised of many other transcription factors that are also critical to the properties of ESCs. We review recent elucidation of a wider transcription network in ESCs.

4 Pluripotency Genomics and Proteomics

In large part, RNA expression marks important differences between two cell types at the molecular level. Several high-throughput technologies are now available to characterize all transcripts expressed in a given cell population. These include sequencing-based approaches, such as EST (expressed sequence

tags) (Boguski et al., 1993; Sharov et al., 2003), SAGE (serial analysis of gene expression) (Richards et al., 2004; Velculescu et al., 1995), and MPSS (massively parallel signature sequencing) (Boheler and Tarasov, 2006; Brenner et al., 2000). Array-based technologies have advanced in parallel. Prominent among commercially made arrays are those manufactured by Affymetrix. These high-throughput technologies, combined with whole genome sequences, have become powerful tools for elucidation of the transcriptome in this, the post-genome project, era. Visionary use of transcriptome data is illustrated by the work of Yamanaka and his colleagues. They used digital differential display (http://www.ncbi.nlm.nih.gov.ezp-prod1.hul.harvard.edu/UniGene/info_ddd.shtml) to compare EST libraries from mES cells and those from various somatic tissues to identify candidates of the LIF/STAT3-independent factor(s). From such data, they identified potentially important ESC-specific factors, including Nanog (Mitsui et al., 2003). From among this set, Yamanaka and his colleagues tested various combinations of factors to reprogram somatic cells to a pluripotent state (Takahashi and Yamanaka, 2006).

In addition to gene expression data, it is now possible to capture more comprehensively the repertoire of proteins in ESCs. Recent development in proteomics research enables the large-scale quantitative analysis of the protein expressed in a given cell. In parallel with the transcriptome which portrays a gene expression profile by microarray, the proteome (PROTEins expressed by genOME) describes the catalogue of the total set of proteins expressed in a cell, an organization, or an organism. The proteome reflects all aspects of cellular proteins, including their synthesis, stability, degradation, and PTMs (posttranslational modifications). Moreover, understanding the interaction of proteins within the context of a cellular network is critical for understanding function. The proteome of ESCs has been characterized using mass spectrometry (MS)-based protein profiling of both undifferentiated and differentiated ESCs (Elliott et al., 2004; Nagano et al., 2005; Van Hoof et al., 2006). These studies and others have generated an extensive set of data for ESCs that serves as an initial protein catalogue complementing mRNA expression data. These data are available for effective comparison of experimental data across different labs. The HUPO (Human Proteome Organization) and the ISSCR (International Society for Stem Cell Research) have established an alliance to provide a platform for collaboration and communication between scientists in each organization (http://www.hupo.org/research/stemcells/).

An alternative approach to revealing protein dynamics in ESCs involves identification of protein complexes, which might be either transient or quite stable. Using affinity purification followed by MS-based peptide microsequencing, Wang et al. characterized putative protein complexes of core pluripotency factors in ECSs (Wang et al., 2006). To enrich for proteins under native conditions, Wang et al. first employed intracellular metabolic biotin tagging to identify proteins physically in association with Nanog. A major advantage of the biotin tagging approach is that it does not rely on the availability of specific antibodies. Often, high-quality antibodies directed to novel proteins are

lacking. As illustrated by Wang et al., affinity purification of individual components of protein complexes can be performed in an iterative fashion. Nanog-associated proteins, either directly or indirectly, are predominantly either transcription factors or others components of the transcriptional machinery. These proteins include previously reported ESCs proteins, such as Oct4 and Dax1. Through iterative purification of complexes following tagging of these proteins and others (Nac1, Zfp281, and Rex1) as second baits, a Nanog-related protein network was generated (Fig. 1). The factors Sall4 and Dax1 have been identified independently by other groups as involved in maintenance of ESCs pluripotency (Elling et al., 2006; Niakan et al., 2006; Sakaki-Yumoto et al., 2006; Wu et al., 2006; Zhang et al., 2006b). Interestingly, these proteins appear interconnected with one another within a large network, suggesting that they function cooperatively in control of gene expression. In addition, proteins of the network are connected to transcriptional co-repressor complexes, such as the NuRD (histone deacetylation) remodeling complex, histone deacetylases, and the PRC1 (polycomb complex 1) (Fig. 1). Both complexes are recently reported as required for pluripotency of ESCs (Endoh et al., 2008; Kaji et al., 2007). These observations are consistent with a model in which proteins of the network operate to silence differentiation as a means for maintaining the pluripotent state. This network, a pluripotency "interactome", provides a framework for exploring additional combinations of factors that permit faithful reprogramming of differentiated cells to an ES cell state.

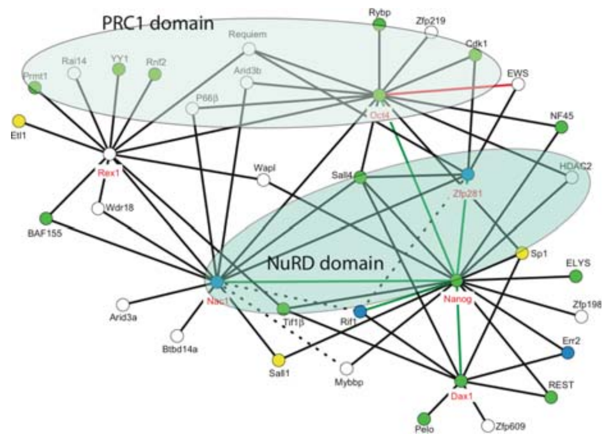

Fig. 1 Depiction of the features of the interactome. Green circles indicate proteins whose knockout results in defects in proliferation and/or survival of the inner cell mass or other aspects of early development; yellow circles are proteins whose knockout results in later developmental defects

5 Extended Transcriptional Network: ChIP-ChIP

How do factors in the pluripotency network function to regulate downstream genes? Genome-wide ChIP (chromatin immunoprecipitation) analysis can be used now to predict target genes controlled by given transcription proteins.

Combination of ChIP and microarray or sequence methods, ChIP-ChIP (Buck and Lieb, 2004), ChIP-PET (paired end ditags) (Loh et al., 2006), and ChIP-Seq (short tag based sequencing) (Barski et al., 2007; Johnson et al., 2007; Mikkelsen et al., 2007; Robertson et al., 2007), are powerful high-throughput methods for identifying gene regions bound by specific proteins. DNA regions bound by Oct4, Nanog, and Sox2 were first identified in both hESCs and mESCs (Boyer et al., 2005; Loh et al., 2006). The principal finding in the initial studies was the remarkable extent of factor co-occupancy at promoter or other gene regions. An extraordinary database of ChIP binding in hESCs and mESCs has been generated recently. Core factors, Oct4, Sox2, and Nanog are also bound to their own promoters. These studies indicate the existence of combinatorial occupancy of target gene promoters by "core" factors within autoregulatory and feed-forward transcriptional circuits. Kim et al. extended these findings further by a modification of the ChIP-ChIP approach, termed bioChIP-ChIP (Kim et al., 2008), that takes advantage of affinity capture of biotinylated proteins bound to chromatin and subsequent hybridization to promoter arrays. By this strategy, factors can be analyzed without the requirement for protein-specific antibodies. Often, protein-specific antibodies fail to perform well in conventional ChIP assays, sometimes for entirely obscure reasons. Kim et al. used the bioChIP-ChIP method to identify the putative target promoters of "core" factors, as well as the somatic cell reprogramming factors (Oct4, Sox2, Klf4, c-myc) and others within the pluripotency network (Nanog, Dax1, Rex1, Zpf281, and Nac1) (Wang et al., 2006). Remarkably, data showed that $\sim$800 gene promoters are bound by four or more transcription factors (Fig. 2a). This finding highlighted greater combinatorial factor binding than previously appreciated. Of the nine factors tested, seven factors (all except c-myc and Rex1) lie within a subgroup sharing many targets and appear to be involved in both activation and repression. Further analysis of the data revealed a striking correlation. The promoters, which are active in ESCs, and then repressed upon differentiation, tend to be occupied by $>$4 factors including Nanog, Sox2, Dax1, Nac1, Oct4, and Klf4. On the other hand, promoters that are expressed upon differentiation but silent in ECSx are generally occupied by few ($<$4) or a single factor. These correlations are illustrated by the gene-set enrichment analyses shown in Fig. 2b. Although the mechanisms accounting for these context-dependent differences remain to be elucidated, these data demonstrate that the same factors function both positively and negatively in transcription.

In addition to transcription factors, histone modifications, specifically H3K4me3 (histone 3 lysine 4 trimethylation) and H3K27me3 (histone 3 lysine 27 trimethylation), signify important aspects of gene regulation in ESCs. c-myc is almost exclusively bound to the promoters with the H3K4me3 signature (Fig. 2c). This finding is consistent with the presence of the H3K4me3 signature at active genes and c-myc functioning as a positive regulator. When other factors (such as Nanog, Oct4 in Fig. 2d) are bound to promoters alone, they do so in association with the H3K27me3 mark, which is correlated with gene silencing. These observations are consistent with a model in which c-myc

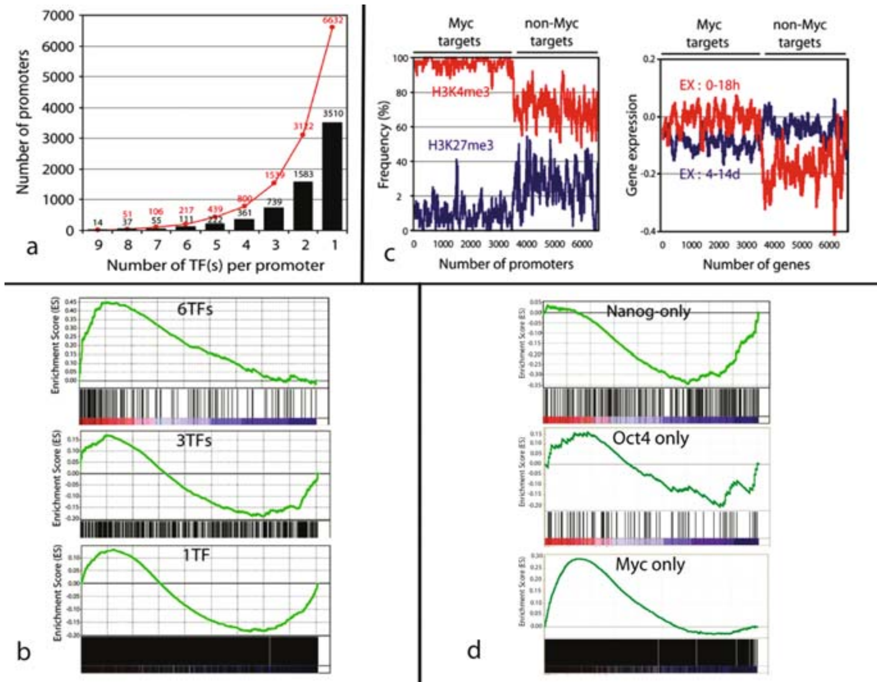

Fig. 2 (**a**)Number of common targets of multiple factors. y-axis represents the number of target promoters occupied by transcription factor(s). Red dots represent the accumulated number of target promoters. (**b**) GSEA (gene-set enrichment analysis) showing the relationship between target gene expression and factor occupancy. Target promoters were classified based on the number of co-occupying factors and corresponding gene expression upon differentiation was tested. Common targets of six factors are enriched in active genes in ES cells, whereas single-factor-only targets are more repressed. (**c**) Left panel: H3K4me3 (red line) and H3K27me3 (blue line) status for Myc target promoters. Right panel: Expression profiles of Myc target genes at different time points upon differentiation (0-18 hr: red, 4-14 days: blue). Total 6632 target genes of any of nine factors are shown, and moving window average (bin size 50 and step size 1) was applied (b and c). (**d**) GSEA analyses showing single-factor Nanog and Oct4 which target more repressed genes, whereas myc alone targets active genes

occupancy reflects broad changes in chromatin accessibility. Such widespread effects may account in part for the potency of c-myc as a facilitator of somatic cell reprogramming. Among the four "Yamanaka" factors, Klf4 may serve in part as an upstream regulator of feed-forward circuits involving Oct4 and Sox2, as well as downstream effectors (e.g., Nanog). Based on the promoter occupancy studies, Klf4 may also regulate c-myc, which itself participates in a feed-forward circuit favoring cell proliferation and chromosome accessibility (Fig. 3). Ng's group in Singapore used ChIP-seq methods with extended 13 transcription factors (Nanog, Oct4, Sox2, Klf4, c-myc, CTCF, Tcfc2I1, n-myc, Smad1, STAT3, Zfx, E2f1, Esrrb) including core factors and reprogramming

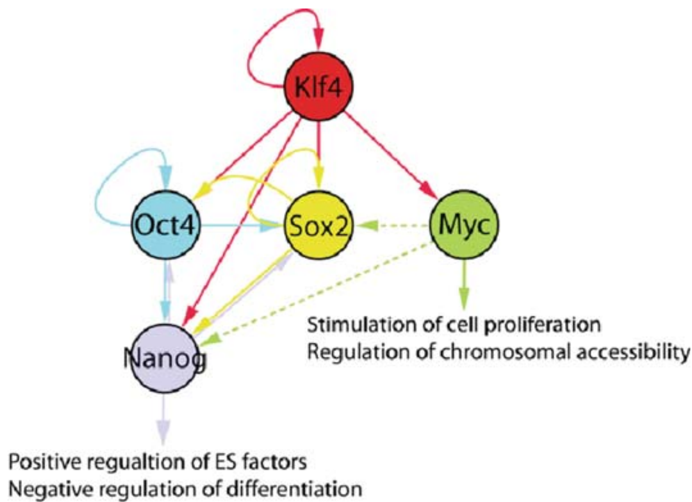

Fig. 3 Transcriptional regulatory circuit within four somatic cell reprogramming factors and Nanog

factors (Chen et al., 2008). ChIP-seq is a variant of ChIP-ChIP that uses high-throughput DNA sequencing rather than tiling arrays for detecting differences. In spite of using different platforms, ChIP-ChIP and ChIP-seq, both reports confirm an extended transcription factor network in mESCs. However, as pointed out above, data have been accumulated by different platforms, such as ChIP-ChIP, ChIP-PET, and ChIP-seq. Different platforms exhibit data variations due to the technical differences in the methods, as well as in data analysis. Comprehensive technological comparisons between different platforms will be most useful in examining these networks in a comprehensive manner (Euskirchen et al., 2007; Mathur et al., 2008).

miRNAs (microRNAs) are a class of short non-coding RNAs. They play a crucial role of posttranscriptional gene regulation. miRNAs have been strongly linked to stem cells, which have a remarkable dual role in development (Foshay and Gallicano, 2007; Hatfield et al., 2005; Kanellopoulou et al., 2005; Murchison et al., 2005; Shcherbata et al., 2006; Stadler and Ruohola-Baker, 2008; Wang et al., 2007b; Yang et al., 2005; Zhang et al., 2006a). Marson et al. used ChIP-seq methods to analyze how miRNA gene expression is controlled by key transcriptional regulators in ESCs (Marson et al., 2008). Interestingly, genome-wide maps of Oct4/Sox2/Nanog and Tcf3 (Cole et al., 2008) occupancy at miRNA promoters are very similar to those of protein-coding genes. There are two sets of miRNAs occupied by Oct4/Sox2/Nanog/Tcf3. One of them is actively expressed in ESCs and the other is silenced in ESCs by polycomb genes in association with the H3K27me3 mark. This result suggests that posttranscriptional control complements transcriptional control in maintaining the pluripotent state.

6 Epigenetics in ESCs

Epigenetics encompasses heritable regulation that is not encoded in the DNA sequence. Epigenetic information may persist over multiple rounds of cell division. Stochastic and environment-induced epigenetic defects are associated with cancer and aging. Several types of epigenetic inheritance systems are involved in retaining "cellular memory" (Ng and Gurdon, 2008). One of the mechanisms is chromatin remodeling with DNA methylation and histone modification (Gan et al., 2007). Chromatin structures play a crucial role for DNA accessibility, which is essential for DNA repair, DNA replication, and gene transcription (Li et al., 2007a).

As noted above, recent technical developments, such as ChIP-ChIP and ChIP-seq, have converted epigenetic research into a high-throughput format to assemble "Chromatin state maps" that describe the genome-wide distribution of chromatin modifications (Mendenhall and Bernstein, 2008). A recent analysis reveals a highly dynamic association of chromatin in pluripotent cells, as compared with that of differentiated cell types (Boyer et al., 2006a).

Genome-wide histone methylation of ESCs has been characterized by several large-scale studies (Ananiev et al., 2008; Barski et al., 2007; Bernstein et al., 2005; Gitan et al., 2002; Guenther et al., 2007; Ikegami et al., 2007; Kim et al., 2005; Mikkelsen et al., 2007; Shiota et al., 2002). Two distinct histone modifications, H3K4me3 and H3K36me3, are known to be related to RNA polymerase II initiation and transcriptional elongation, respectively (Sparmann and van Lohuizen, 2006). The map of H3K36me3 is strongly correlated with RNA expression, whereas not all promoters associated with H3K4me3 are active in ESCs. H3K4me3 marks are not only seen in CG-rich promoters of many ubiquitously expressed housekeeping genes, but also found to correspond to developmental regulators and signaling protein genes that are not expressed in ESCs. Interestingly, these inactive promoters with H3K4me3 marks also bear the repressive H4K27me3 mark, forming the "bivalent" state (Bernstein et al., 2006; Boyer et al., 2006b). Silencing activity seems dominant over activation at "bivalent" targets. About 22% of CpG-rich promoters are bivalently marked in mESCs. However, bivalent marks are reduced upon terminal differentiation, resolving into single marks or no identified marks. In committed cells, inactive genes are often marked by H3K27me3, rather than bivalent signatures, perhaps in association with lineage choice and commitment. Bivalent marks have been proposed to provide flexibility in the decision of a gene to be activated or repressed. The "bivalent" chromatin state may poise genes for the subsequent activation during lineage-specific differentiation. In this sense, the "poised state" has been suggested to be critical for maintaining the pluripotency in ESCs. The extent to which the "bivalent" state is a distinctive characteristic of ESCs (or other stem cells), as opposed to other cell types, and to which it is an essential component in maintenance of pluripotency are unresolved.

TrxG (Trithorax-group) and PcG (polycomb-group) proteins have an important role in cellular memory system. H3K4me3 is catalyzed by the MLL (mixed-lineage leukemia protein) component of the TrxG complex, and H3K27me3 is catalyzed by the Ezh1/2 component of the PRC2 (PcG 2 complex) (Cao et al., 2002; Francis and Kingston, 2001; Simon and Tamkun, 2002). The PRC2 complex contains at least four subunits, including the three PcG proteins EZH2, EED, and SUZ12 and the histone-binding proteins RbAp48/46. A second PcG complex, PRC1, which likely exists in many variant forms due to a large number of homologues in mammalian cells, contains at least six different subunits: the polyhomeotic- (HPH1-3), polycomb-/CBX (HPC1/CBx2, HPC2/CBX4, HPC3/CBX8, CBX6, CBX7), the RING1- and 2- (RING1A/B), the posterior sex comb- (BMI1, MEL18, MBLR, and NSPC1), and sex comb on midleg (SCML1-2) proteins (Levine et al., 2002). PRC1 is thought to bind methylated H3K27 and mediate stable silencing through recruitment of DNA methylation complexes and chromatin compaction. Gene knockout studies demonstrated the importance of PcG complexes for proper development. Deletion of any of the PRC2 members results in embryonic lethality (Faust et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004). However, EED-, Ezh2-, and SUZ12-deficient ESCs retain some degree of pluripotency (Boyer et al., 2006b; Chamberlain et al., 2008; Morin-Kensicki et al., 2001; Pasini et al., 2004; Shen et al., in press). These data suggest that PRC2 may be dispensable for overall maintenance of pluripotency. PRC1 members play important role during later stage of development except Ubiquitin E3 ligase Ring1b. Deletion of either EDR1 or Bmi1 results in loss of hematopoietic stem cells. Loss of Bmi1 also impairs neural and mammary stem cells. On the other hand, deletion of Ring1b is embryonic lethal (Voncken et al., 2003) and Ring1b appears to be involved in maintenance of ESCs by repressing high CpG content promoters and bivalent histone marks or active H3K4me3 marks (van der Stoop et al., 2008)

The recent discovery of histone demethylases further highlights the flexibility of epigenetic modifications. The UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome) and JMJD3 serve as H3K27 demethylases (Hong et al., 2007; Loh et al., 2007). The Jmj C (Jumonji C)-containing JARID1 protein family including the RBP2/JARID1A (RB-binding protein 2), PLU1/JARID1B, and SMCX/JARID1C serve as a demethylases with specificity for tri- and dimethylated H3K4 (Christensen et al., 2007). The LSD1 (lysine-specific demethylase1) serves as a dimethylase for mono- and dimethylated H3K4 when in complex with CoREST to silence gene expression (Shi et al., 2004) and serves as a demethylase for mono- and dimethylated H3K9 when in complex with AR (androgen receptor) to promote gene activation (Metzger et al., 2005). Jmjd1a (also known as JHDM2A (JmjC-domain-containing histone demethylase2)) also demethylates H3K9 mono- and dimethylation in vitro and functions as a coactivator for AR to demethylate chromatin of AR target genes (Yamane et al., 2006). However, LSD1 removes the methyl group through a flavin-adenine-dinucleotide-dependent oxidation reaction (Shi

et al., 2004), whereas JmjC-domain-containing proteins remove methyl groups through a hydroxylation reaction that requires alpha-ketoglutarate and Fe(II) as cofactors (Tsukada et al., 2006). Jmjd2c has a different specificity and is shown to convert H3K9 and H3K36 from trimethylation to dimethylation (Whetstine et al., 2006). Loh et al demonstrated that Jmjd1 and Jmjd2c expression are under Oct4 regulation. Either Jmjd1a or Jmjd2c depletion induces ESCs differentiation (Loh et al., 2007). This suggests that histone demethylases may also play important role in the ESCs transcriptional network and may specify the epigenetic status of pluripotency-associated genes.

7 State of ESCs

We have broadly reviewed the state of pluripotency from multiple perspectives: extrinsic factors, transcription factors, protein complexes, and the genome-wide chromatin state. In just a few years, investigators have generated extensive resources to address the pluripotent state. It is evident that the pluripotent state is not static, but highly dynamic. Pluripotency may depend on the extent to which cells may be maintained in a "poised state", reflecting the balance between proliferation and differentiation. A principal distinction between ESCs and adult stem cells relates to their modes of cell division. Adult stem cells are believed to divide asymmetrically in order to generate two cell types, one committed to self-renewal and the other to differentiation. In contrast, ESCs divide symmetrically to produce identical daughters. The progenitors arising from a symmetrical division may subsequently choose to remain pluripotent or differentiate. For example, Nanog is indispensable for early development, however, its expression is surprisingly dynamic among ESCs. Hence, ESCs appear metastable, balancing between two states, self-renewal and pro-differentiation. Unlike adult stem cells, which only need to supply a limited cell type, this metastable nature of ESCs has an advantage in early embryogenesis, which necessitates generating diverse cell types in a short period. Presently, many investigators seek to differentiate ESCs to specific cell lineages. However, it is hard to define conditions to differentiate ESCs toward an uniform cell type. This metastable nature of ESCs, which may have an advantage in embryogenesis, complexes isolation of pure cell type in vitro. The state of pluripotency is highly dynamic. Further mechanistic understanding of pluripotency may facilitate use of ESCs as potential cellular sources in regeneration medicine and independently stimulate comparisons of pluripotency and the malignant phenotype.

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MicroRNAs in Stem Cells and Cancer Stem Cells

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Abstract MicroRNAs (miRNAs) have been shown to play a role in the development, cell division, proliferation, maintenance, and differentiation of stem cells (embryonic and adult) and in tumorigenesis, cancer cell migration, and metastasis, and this list continues to grow. In this chapter, we review various aspects of miRNA biology, including its biogenesis and miRNA–protein complexes. We will look at the recent development into the mechanism of its functions and the role of miRNA in stem cells and various cancers. We discuss some of the open questions in the field and the prospect of a potential role of miRNAs in cancer or tumor-initiating stem cells. We also comment on budding but promising therapeutic application of miRNAs in pathological scenario. Understanding this layer of regulation by miRNA will uncover many interesting avenues in future in learning the biology of life.

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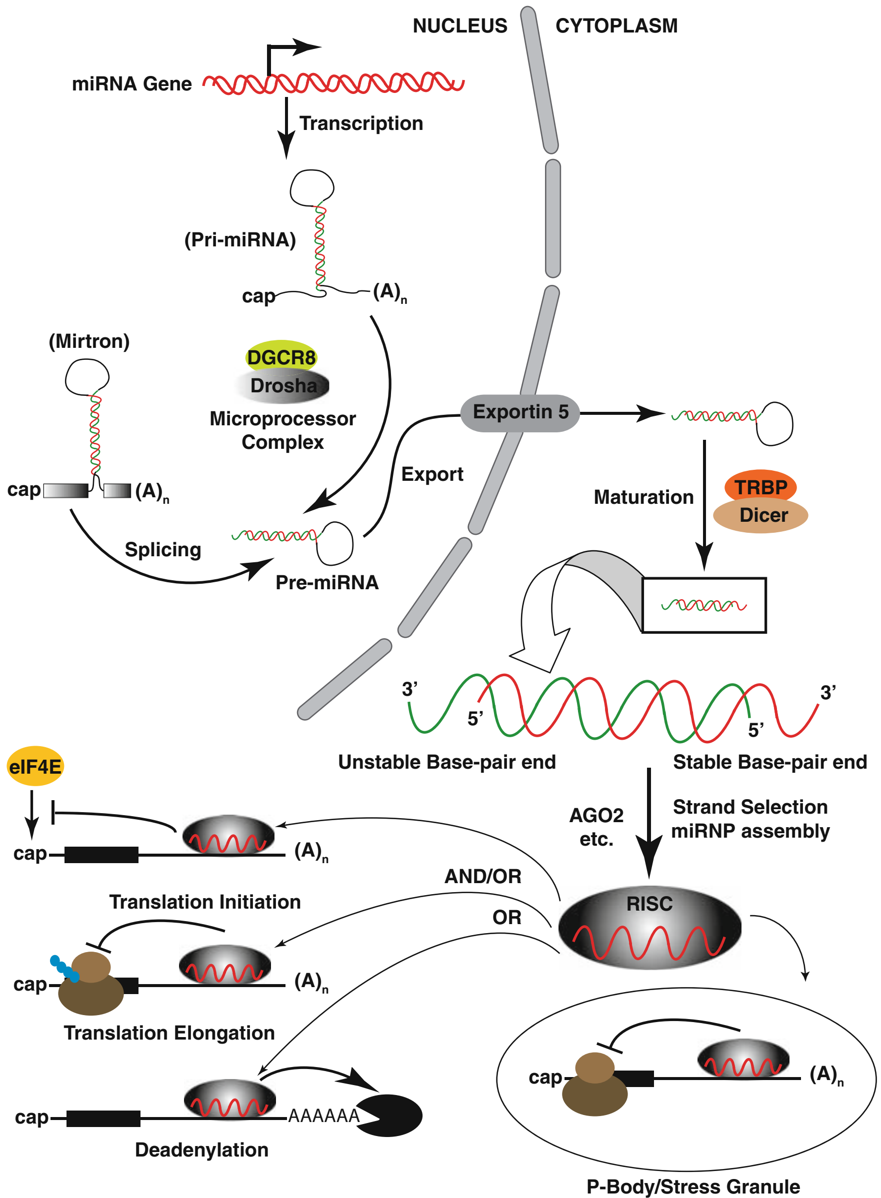
1 Biogenesis of miRNAs

The field of RNA biology has come a long way since the early days when RNA was considered to be just a passive messenger, carrying genetic information from DNA to proteins (Crick, 1970). Early evidence for RNA being actively involved in the regulation of gene expression can be traced back to 1961, when it was suggested that RNA molecules can inhibit the expression of operons by blocking operator function through base pairing (Jacob and Monod, 1961). However, one of the most exciting developments in the study of RNA took place about a decade ago, when microRNAs (miRNAs) were discovered (Lee et al., 1993; Wightman et al., 1993). The miRNA database (miRBase: http://microrna.sanger.ac.uk/sequences/) currently lists around 3097 mammalian miRNAs, with 678 miRNAs identified in the human genome (version 11, April 2008). The database has seen a dramatic increase in the number of listed miRNAs in various systems (from 218 in 2002 to 6396 in 2008). While most miRNAs are transcribed by RNA polII (Cai et al., 2004; Lee et al., 2004), some, such as the cluster of miRNAs on chromosome 19, can be transcribed by RNA polIII (Cai et al., 2004). Based on their location in the genome, many miRNA genes are transcribed as polycistronic transcripts, as they tend to be clustered together (Lee et al., 2004). The physical location of miRNA genes can be in various types of transcriptional units: in the introns of protein-coding and non-protein-coding genes and in the exons of non-protein-coding genes (Rodriguez et al., 2004). It has also been reported that when an miRNA gene is in the imprinted locus of the genome, its expression is parent specific, e.g., mir-127 and mir-136 (Seitz et al., 2003).

The primary miRNA transcripts (pri-miRNAs) form short double-stranded hairpin structures and are processed by endoribonucleases and their associated proteins in the nucleus and cytoplasm. In the nucleus, the endoribonuclease Drosha and its interacting protein DGCR8 (microprocessor complex) excise the stem loop of approximately 70 nucleotides with a 2-nucleotide (nt) 3′ overhang from the pri-miRNA transcript (Lee et al., 2002). However, the discovery of a pre-miRNA/intron (mirtrons) suggests that there is at least one alternative mechanism of miRNA biogenesis that does not require microprocessor activity for its maturation. The mirtrons are products of debranched introns that can fold to have a stem-loop structure and contain a 5′ monophosphate and 3′ 2-nt overhang (similar to microprocessor-produced pre-miRNAs) (Okamura et al., 2007; Ruby et al., 2007). The stem loop processed by the microprocessor complex (pre-miRNA) and processed mirtron is then exported out of the nucleus through a RanGTP and Exportin-5 complex in a GTP-dependent manner (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). In cytoplasm, the pre-miRNA stem loop is further processed by another endoribonuclease, Dicer, which recognizes the 2-nt overhang on the 3′ end of the pre-miRNA generated by Drosha activity. The N-terminal PAZ and c-terminal dsRNA-binding domains of Dicer are responsible for its interaction with pre-miRNA (Zhang et al., 2004). After Dicer forms a complex with pre-miRNA, the two

RNase III domains of Dicer undergo intra-molecular dimerization to form a single cleavage center and cleave the pre-miRNA separated by 2 nt roughly about 22 nt away from the termini, simultaneously generating a 3′ 2-nt overhang (Zhang et al., 2004). After Dicer activity, the mature miRNA intermediate has a 2-nt 3′ overhang on either end of the double-stranded duplex (corresponding to the stem of the pre-miRNA) (Bernstein et al., 2001) (Fig. 1).



1.1 The miRNA–Protein Complex

Functional miRNA is a miRNA–ribonucleoprotein complex, also known as the miRNA- RNA-induced silencing complex (miRNA–RISC). The miRNA duplex, after Dicer activity, unwinds and binds to Argonaute (Ago) protein to form the core of the functional effector complex of miRNA, the miRNP (Mourelatos et al., 2002). Although the events that orchestrate this loading of miRNA onto Ago protein are still not very clear in mammals, studies from various model systems have provided crucial insight into this process. Studies from *Drosophila melanogaster* have elucidated a thermodynamic basis for the determination of which of the two miRNA strands will actually be in complex with Ago. According to this model, the strand with lower thermodynamically stable base pairing with the opposite strand on its 5′ end is the mature miRNA (Tomari et al., 2004; Tomari and Zamore, 2005). In flies, this thermodynamic stability is sensed by a heterodimer of Dcr-2/R2D2, where R2D2 have selectively higher affinity toward the more stable end of the duplex (Tomari et al., 2004).

Further processing/removal of the non-miRNA strand from the duplex and loading of the miRNA into Ago protein is orchestrated through a bypass mechanism, the details of which are still unclear. There are four Argonaute families of proteins in mammals (AGO1–AGO4), and only AGO2 has been shown to be functional in RNA interference. The RNaseH-like PIWI (P-element-induced wimpy testis) domain of AGO2 is capable of cleaving the target RNA at the center of the siRNA–mRNA paired region (Liu et al., 2004; Meister et al., 2004b; Pillai et al., 2004). Apart from Argonaute, the miRNP complex consists of other proteins that may facilitate different aspects of miRNA function (Peters and Meister, 2007). The interactions between miRNP complex and its target mRNA are driven by specific base-pairing interactions between miRNA and sequences located in the 3′ UTR of target mRNA. The nucleotides between

Fig. 1 MicroRNA biogenesis and RISC assembly. A series of post-transcriptional processing events are involved in biogenesis of miRNAs. The microprocessor complex (Drosha (RNase III type of endonuclease) and DGCR8) recognizes the hairpin structures formed by pri-miRNAs and produce an approximately 70-nucleotides hairpin known as pre-miRNA. Notable deviation from this step of biogenesis is “mirtrons,” a class of miRNAs which do not require microprocessor complex and are processed by the splicing machinery. The end product of spliced out introns (mirtrons) have hairpin structures similar to that of pre-miRNAs. These pre-miRNAs (~70 nucleotides in length) are then transported to cytoplasm by exportin-5, where they are processed to ~22 bp miRNA duplex by Dicer–TRBP complex. These 22 bp duplexes have 3′ overhangs of 2 nucleotides. One strand of these duplexes is selectively assembled into RISC (*R*NA-*I*nduced *S*ilencing *C*omplex), while the other strand is degraded. Both Dicer-mediated cleavage and RISC assembly can be coupled, although this step is not clear at the moment. This ribonucleoprotein complex (RISC) is the functional unit and participates in miRNA–mRNA hybrid duplex formation. The miRNP-mediated regulation of gene expression involves blockade of translation inhibition, blockade of translation elongation, mRNA deadenylation and sequestration of mRNA-miRNP complex into P-bodies or stress granules

positions 2 and 8 (seed sequences/region) must be perfectly base-paired to start an interaction between miRNP and mRNA (Brennecke et al., 2005; Doench and Sharp, 2004; Grimson et al., 2007; Lewis et al., 2005; Nielsen et al., 2007).

2 Mechanisms of miRNA Function

miRNAs regulate the translation or degradation of target mRNAs by forming a partial hybrid duplex at their cognate target sequences in the 3′ UTRs of target mRNAs. There are two different ways miRNAs can influence gene expression as discussed below (Fig. 1).

2.1 Translational Repression

There is now evidence suggesting that miRNP-mediated repression of translation might occur both before and after the initiation of translation.

2.1.1 Pre-initiation Repression of Translation

The presence of the m^7G cap at the 5′ end of mRNAs is essential for miRNA-mediated repression of translation initiation (Humphreys et al., 2005; Pillai et al., 2005). This requirement was further proven by bi-cistronic reporter assays and polysome gradient analyses. In bi-cistronic reporter assays (where one cistron was placed under cap and the other under IRES (internal ribosome entry site)), it was shown that only the cap-dependent cistron was specifically repressed. In polysome gradient experiments, it was shown that the reporter mRNAs with miRNA target sequences or artificially tethered AGO2 protein at the 3′ UTR had low sedimentation coefficients, suggesting a lack of translational initiation. In another mechanism, the AGO2 protein plays a central role, where it has been proposed to compete for m^7G cap with eI4E (a cap-recognizing translation initiation factor). The central domain of AGO2 protein has sequence similarities with eI4E, and when mutated AGO2 is unable to mediate translational repression, even when artificially tethered to the 3′ UTR sequences (Kiriakidou et al., 2007). This observation may further explain why the presence of multiple miRNA-binding sites in the 3′ UTR is able to mediate a robust miRNA-mediated repression of translation. It is conceivable that the presence of multiple low-cap-affinity AGO2 proteins at the 3′ UTR is a more efficient inhibitor of eI4E binding at the cap. It was further established that mRNAs to be targeted by miRNA-mediated inhibition of translation should also have a poly(A) tail (Wakiyama et al., 2007). The inhibition of assembly of 60S and 40S ribosomal subunits has also been proposed as a mechanism of miRNA-mediated translation inhibition. It was shown that the AGO2-Dicer-TRBP complex interacts with eIF6 and 60S ribosomal subunits. Partial depletion of eIF6 in cells leads to rescue of miRNA-mediated repression of target mRNAs (Chendrimada et al., 2007).

2.1.2 Post-initiation Repression of Translation

Although most studies suggest that miRNA-mediated repression of translation occurs at the translation initiation step, the presence of miRNP–mRNA in the polysome fraction is not consistent with the inhibition of initiation model. It was shown that lin-14 and lin-28 mRNAs (targets of lin-4 miRNA) remain associated with polysome at different developmental stages (Olsen and Ambros, 1999; Seggerson et al., 2002). These observations are now being challenged, however, as the presence of these miRNP–mRNAs in the polysome fraction might not be enough to suggest that these were, in fact, stalled complexes and that translation had already been initiated.

2.2 Destabilization of mRNA (mRNA Deadenylation and Decay)

The levels of miRNA-regulated mRNAs have been shown to decrease in many studies, and initial suggestions that levels of miRNA-regulated mRNAs remain unchanged were only partially correct. Degradation of mRNA is triggered by shortening of the poly(A) tail (deadenylation) and is followed by either 3′–5′ exonuclease cleavage of mRNA or decapping and 5′–3′ degradation by exonuclease (XRN1) (Parker and Song, 2004). A conserved interaction between *D. melanogaster* GW182 and AGO1 protein has been proposed to be crucial for marking miRNA-interacting mRNA for degradation (Behm-Ansmant et al., 2006). The GW182 then recruits components of the deadenylation complex CCR4-CAF1-NOT to the miRNA-regulated mRNAs. At this stage, if the decapping enzymes DCP1 and 2 are missing, there is an accumulation of deadenylation mRNAs and a decrease in miRNA-mediated degradation of messages (Behm-Ansmant et al., 2006; Eulalio et al., 2007b). It is unclear, however, if these deadenylated mRNA further participate in translation inhibition mechanisms. It is possible that the mechanisms of both translation inhibition and decay work synergistically at this stage for effective miRNA-mediated regulation.

2.3 Sequestration of mRNA into Sub-cellular Locations

Sub-cellular locations such as P-bodies and stress granules have been known to be accumulation sites for translationally repressed mRNA–protein complexes (Eulalio et al., 2007a). There is evidence to suggest that mRNAs regulated by miRNA-mediated translational repression accumulate in P-bodies. The inhibition of miRNA biogenesis or depletion of proteins involved in miRNA function leads to loss of visible P-bodies in *D. melanogaster*. Studies from various organisms suggest that the resident P-body protein, such as RCK/p54, 4E-T, Pat1, and RAP55, may have an inhibitory effect on translation initiation (Filipowicz et al., 2008). This sequestration of miRNA-repressed mRNAs in P-bodies can also be partially attributed to the reversibility of miRNA action. It has been shown in human hepatoma cells that after amino acid starvation or

other types of stress these miRNA-repressed mRNAs were able to come out of P-bodies (in a ELAVL1-dependent mechanism) and get recruited to polysomes (Huang et al., 2007).

3 Stem Cells and miRNA

Metazoans depend on three classes of stem cells, namely, embryonic stem cells, adult stem cells, and adult germline stem cells, for their early development and the maintenance of organs/tissues during adulthood. Stem cells have the unique property of self-renewal, defined as indefinite propagation, while retaining their potential to differentiate into multiple lineages when appropriate signals are provided both in vitro and in vivo. Their "stemness" is maintained through a series of intricate mechanisms, including a network of transcription factors, an internal epigenetic program, and extrinsic signals from the surrounding niche. Since their discovery in *C. elegans,* where miRNAs were shown to play an important role in development, miRNAs have been shown to play a critical role in the maintenance of self-renewing stem cells and in their ability to differentiate into various lineages (Cheng et al., 2005b; Forstemann et al., 2005; Hatfield et al., 2005; Houbaviy et al., 2003; Kanellopoulou et al., 2005; Kuwabara et al., 2004; Lee et al., 2005; Murchison et al., 2005; Suh et al., 2004; Tang et al., 2006; Yang et al., 2001).

3.1 Embryonic Stem Cells and miRNA

Embryonic stem cells are a distinct set of pluripotent stem cells from the early embryonic developmental stage (from the inner cell mass of blastocysts). They can be propagated indefinitely while maintaining their plasticity under in vitro conditions and differentiate into all three germ layers of a developing animal. The importance of miRNAs in normal development was illustrated when disruption of Dicer-1 in *D. melanogaster* and of Dicer in mice led to embryonic lethality at day 7.5 (Bernstein et al., 2003; Kloosterman and Plasterk, 2006; Murchison et al., 2005; Wienholds and Plasterk, 2005; Yang et al., 2005). A close study of these abnormal embryos showed a loss of pluripotent stem cells (Bernstein et al., 2003). When Dicer function was disrupted by gene targeting in mouse ES cells, it resulted in ESCs with defective miRNA processing and compromised differentiation capabilities (Bernstein et al., 2003; Kanellopoulou et al., 2005). These cells also elucidated a role for centromeric repeat-specific small RNAs, whose expression is dependent on Dicer function, in the maintenance of the heterochromatin status of these repeats. Dicer deletion/ablation in specific tissues also suggests the critical role of miRNA biogenesis in their development and differentiation. Conditional Dicer-knockout mice in which the Dicer gene was deleted specifically in the primordial germ cells (PGCs) and spermatogonia exhibited reduced proliferation resulting in retarded

spermatogenesis (Hayashi et al., 2008). A recent report also showed that Dicer deletion in the mesenchyme of the Mullerian duct in the female reproductive tract caused sterility, suggesting that miRNA biogenesis is required for proper Mullerian duct differentiation (Nagaraja et al., 2008). Conditional inactivation of Dicer in the retina led to progressive degeneration, and the animals were unable to respond to light, suggesting that miRNAs are involved in retinal neurodegenerative disorders (Damiani et al., 2008). Selective inactivation of Dicer in the forebrain neurons caused cellular and tissue morphogenesis defects, suggesting a role for miRNA in neurological disorders (Davis et al., 2008). It would be interesting to look at Dicer ablation selectively in neural stem/progenitor populations in embryos and adults. Deleting Dicer in different tissues, such as the heart and B and T cells, also indicates the importance of miRNA at multiple layers (Chen et al., 2008; Koralov et al., 2008).

Another conditional mutation study where DGCR8 was mutated showed a decreased differentiation potential in the resulting mutant ES cells (Wang et al., 2007). Results from these mutation studies clearly showed a critical role for miRNAs in the differentiation of ES cells. Cloning efforts to identify ES cell-specific miRNA revealed that six miRNAs, miR-290–295, are specifically expressed in ES cells and could be involved in the maintenance of pluripotency (Houbaviy et al., 2003), as their expression levels decreased significantly upon onset of differentiation. A similar study where miRNAs specific for human ESCs were cloned also identified two clusters of miRNAs (miR-371, -372, -373, and -373* on chromosome 19 and miR-302, -302b*, -302c, -302c*, -302a, -302a*, -302d, and 367 on chromosome 4) and showed that expression of these miRNAs was down-regulated upon differentiation (Suh et al., 2004). The presence of these ES cell-specific miRNAs in conserved clusters may perhaps make their coordinated expression and repression based on cellular intrinsic or extrinsic cues possible. There is evidence that these miRNAs do, in fact, better reflect ES cell status compared to expression levels of Oct4 mRNA (Palmieri et al., 1994; Yeom et al., 2006). miR-302 is noteworthy, as it is expressed in mouse ESCs, hESCs, and human embryonic carcinoma cells.

As evidenced by the loss-of-function experiments with Dicer and DGCR8, a more direct role for miRNA is perhaps in the regulation/facilitation of differentiation of ES cells. There are, in fact, a set of miRNAs whose expression goes up when ES cells start to differentiate (e.g., miR-21) (Houbaviy et al., 2003; Singh et al., 2008). It is well established now that the pluripotency of ESCs is preserved by an interconnected network of transcription factors. A network of miRNAs responsible for maintaining the identity of a specific cell type has been documented (Johnston et al., 2005; Tsang et al., 2007). It is apparent now that miRNAs can act as another layer of factors that can influence gene expression patterns. It will be interesting to see how the inter-connection between the transcription factor and miRNA networks regulates the self-renewal, differentiation/lineage potential of ESCs. In one such study, the network of core transcription factors (Oct4, Sox2, Nanog, and TCF3) known to maintain embryonic stem cells in a self-renewing pluripotent state was also shown to

regulate expression of a group of miRNAs (Boyer et al., 2005, 2006; Cole et al., 2008). These miRNAs (miR-290–295 cluster), in turn, contribute to the maintenance of ESC identity and yet help to keep ES cells ready for controlled and efficient differentiation. A network between transcription factors and miRNA can now be generated, where miRNAs work in an incoherent feed-forward regulation and help maintain or fine-tune a steady-state level of core transcription factor target genes. For example, core transcription factors were shown to co-occupy and activate expression of Lefty1 and Lefty2, and at the same time the expression of these genes was fine-tuned through core transcription factor-mediated expression of miRNAs (Cole et al., 2008; Marson et al., 2008). This group of miRNAs can target the 3′ UTRs of Lefty1 and Lefty2. The core transcription factors, along with the polycomb complex, were shown to co-occupy the promoters of this group of miRNA genes, whose transcripts are largely missing in ES cells.

As the ES cells are kept in a poised state compatible for efficient differentiation, it was shown that the maturation step of a specific group of miRNAs was blocked in ES cells in an incoherent feed-forward regulation fashion (Fig. 2).

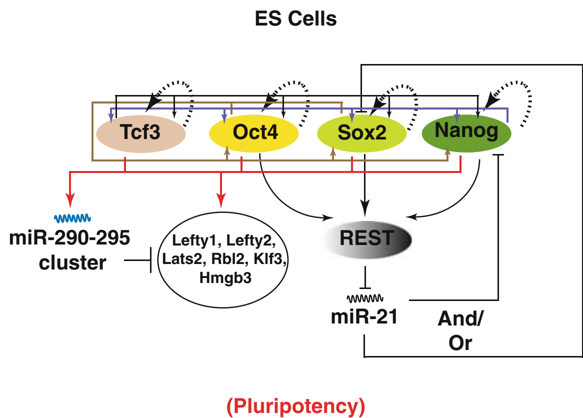


Fig. 2 The network factors in maintenance of embryonic stem cells identity. The core transcription factors (Oct4, Sox2, Nanog, and Tcf3) are known to regulate expression of their own genes apart from multiple other genes in embryonic stem cells and are key factors for maintenance of self-renewal and pluripotency. There are a group of miRNA genes which are directly regulated by the core transcription factors. Depending on co-factors associated with the core transcription factors the miRNA genes are either expressed, e.g., miR-290–295 cluster (shown here) or repressed (not shown). The miR-290–295 cluster of miRNAs then in turn regulate expression of various factors including Lefty1 and Lefty2 and help in maintenance of a differentiation-poised state of ESCs (an example of incoherent feed-forward regulation (Marson et al., 2008)). On the other hand Oct4, Sox2, and Nanog have been shown to co-occupy REST gene, a transcriptional repressor highly expressed in ESCs. REST represses expression of multiple miRNA genes including that of miR-21 (see text). The repression of miR-21 is important for maintenance of core transcription factor network as it can potentially target expression of Sox2 and/or Nanog

Where it was shown that mature let-7 levels were very low in ES cells, pre-let-7 was quite abundant. Mechanistically, the presence of Lin-28 (also regulated by core transcription factors) blocks the maturation of let-7. Thus, the fact that ES cells are poised for efficient differentiation could be partly due to the presence of precursor miRNAs, which can quickly become mature effector miRNAs upon the onset of the differentiation program (e.g., let-7 g) (Viswanathan et al., 2008). Apart from their collective roles in embryonic stem cell biology (miRNA biogenesis-deficient ES cells), individual miRNAs have been shown to play a role in lineage restriction, self-renewal, etc.

In our studies, we found a link between a transcription repressor (REST/NRSF) and miRNA (miR-21) in the maintenance of self-renewal in mouse embryonic stem cells (Singh et al., 2008). Although controversial at present, with evidence in support and against it (Jorgenson et al., 2009, Buckley et al., 2009, Singh et al., 2009), we found that REST blocks expression of miR-21 when cells are grown under self-renewal conditions. REST itself is quickly down-regulated by proteasome-mediated degradation upon the onset of differentiation (Ballas et al., 2005). This decrease in REST levels, in turn, leads to increased expression of miR-21. The putative targets for miR-21, which are key components of the network of transcription factors responsible for maintenance of self-renewal in ES cells, include Sox2 and/or Nanog (Fig. 2). When overexpressed in ES cells growing in the presence of LIF, miR-21 was able to down-regulate self-renewal efficiency and the expression levels of various self-renewal markers, including Sox2 and Nanog. The proposed model (see Singh et al., 2008) is a reflection of how intricate networks of transcription factors and miRNAs may co-operate to maintain self-renewal in ES cells (Singh et al., 2008). In another example, when miR-134 (normally expressed in high levels in the adult central nervous system) is overexpressed in ES cells it leads to its differentiation toward ectodermal lineage and is able to block/overcome the signal from LIF in the media. It was also shown that miR-134 is able to directly target Nanog and LRH1 (Tay et al., 2008). One of the objectives of studying mES cells is to better understand the underlying mechanisms of normal development and differentiation. In vitro protocols now can guide the ES cells to differentiate into specific lineages. In one such study, it was discovered that miR-133b (a mid-brain-specific miRNA) can negatively regulate the function of ES cell-derived dopaminergic neurons by blocking Pitx3 and regulating dopamine synthesis (Kim et al., 2007).

4 The Role of miRNAs in Gene Expression

The role of transcription factors in the regulation of gene expression is well established, and our understanding of how miRNAs regulate gene expression is increasing. These two factors co-operate to establish a specific cell identity, and they are remarkably similar at the mechanistic level (Hobert, 2008). Both transcription factors and miRNAs have been shown to be pleiotropic and to co-operate with different partners in a context-dependent manner; their

accessibility to the target is dependent on the physical state of the target; they are subjected to post-transcriptional regulation; and they are capable of working as an integral part of networks (Buck and Lieb, 2006; Grimson et al., 2007; Kawahara et al., 2007; Kedde et al., 2007; Lee et al., 2008; Robins et al., 2005; Saetrom et al., 2007; Thomson et al., 2006). Apart from these similarities, miRNA differ from transcription factors in many ways that perhaps allow miRNAs to work in a wide range of cellular processes in a temporal and context-dependent manner. The repressive function of miRNAs is restricted by the availability of target mRNAs and the length of the 3′ UTR of the target mRNAs; thus, miRNAs function to fine-tune the existing gene expression program in the cell and can only influence a sub-set of transcription-factor-regulated processes (Hobert, 2008). Because miRNAs function to block protein translation and can be located sub-cellularly, and their repression can be reversible, they are better suited to work as more rapid effector molecules in response to changing cellular environments and to maintain homeostasis, as compared to transcription factors (Ashraf et al., 2006; Bhattacharyya et al., 2006; Martin et al., 2000; Schratt et al., 2006).

5 MicroRNAs and Cancer

Mechanisms that are known to be responsible for changing the expression patterns of tumor suppressor genes and oncogenes have also been implicated in altering the expression patterns of miRNAs. Such mechanisms include wide-scale genomic aberrations, mutations affecting expression and processing, and epigenetic alterations. For miRNA, the mutations affecting target interactions will also have a loss-of-function effect (Cowland et al., 2007).

The genomic locations of many miRNA genes are areas of chromosomes that frequently undergo rearrangement, amplification, and deletion in various types of cancers (Calin et al., 2004). The identification of miRNA genes in such chromosome regions may explain the corresponding absence of any known or putative oncogenes or tumor suppressor genes in these regions, which appear to be important in cancers. The expression of miRNAs can be either up-regulated (these miRNAs can function as oncogenes and are called onco-miRs) or down-regulated (these miRNAs can function as tumor suppressors and are called tumor suppressor miRs) (Costinean et al., 2006). This classification of miRNAs as tumor suppressors or oncogenes is based on their effect on the target genes, as mechanistically the miRNAs are repressor molecules only (Fig. 3). For example, if the target of miRNA is a known tumor suppressor, the miRNA is an onco-miR. Conversely, if the target is an oncogene, the miRNA is a tumor suppressor miR. It is noteworthy that the same miRNA can be an onco-miR or a tumor suppressor miR, depending on its tissue-specific targets. It has been shown that in cases of chronic lymphocytic leukemia (CLL) miR-15a and mir-16-1 genes are either deleted or down-regulated. These alterations in the

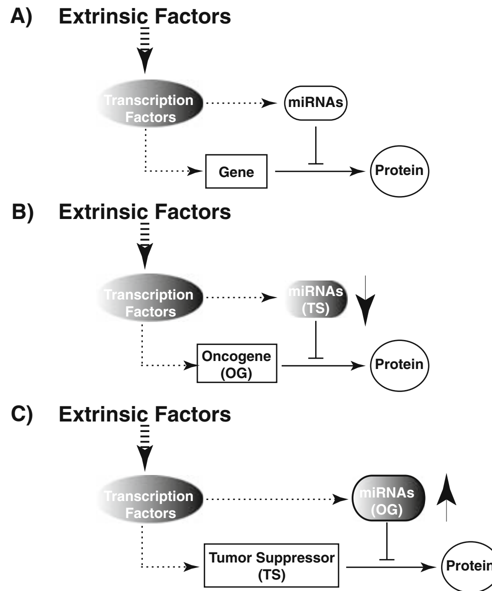


Fig. 3 Cancer and miRNA. The cellular identity is maintained by expression of a network of transcription factors (partly in response to extrinsic factors) and their contribution to the signature expression profiles of protein-coding genes as well as miRNAs. The *arrows with broken lines* represent either activation or repression of target genes by a network of transcription factors. **(A)** Regulation of miRNAs and their involvement in incoherent feed-forward regulation (observed in embryonic stem cells, Fig. 2) (Marson et al., 2008). **(B)** If the targets of miRNAs are oncogenes (OG), the miRNAs are defined as a tumor suppressor (TS) miRNAs. Loss of function for these miRNAs (shown by *downward arrow*) thus may result in tumor formation. (C) If the targets of miRNAs include tumor suppressor genes, these miRNAs are defined as oncogenic miRNAs or onco-miRs. Gain of function of these miRNAs (shown by *upward arrow*) may thus result in tumor formation

expression of miRNAs have been proposed as one of the early events in the pathogenesis of CLL (Calin et al., 2002).

In recent years, the expression profiling of miRNA genes has emerged as a potent tool for classification, diagnosis, grading, prognosis, and treatment outcome assessment in various cancers (Calin and Croce, 2006; Calin et al., 2005; Yanaihara et al., 2006). Stage I lung cancer has been shown to be tightly correlated with the expression profile of a small group of miRNA genes (Yanaihara et al., 2006). Expression profiles of miRNAs in CLL can distinguish between benign and aggressive tumors. Processing defects due to mutations in precursor miRNA molecules lead to the deregulation of specific miRNAs in CLL (Calin et al., 2005). The amplification of the region harboring a miRNA gene, loss of or deregulation of transcription factors, and loss of methylated CpG islands in promoter regions may lead to activation of miRNA expression. Chromosomal translocation in chromosome 11q23 in acute lymphoblastic

leukemia (ALL) or acute myeloblastic leukemia (AML) results in fusion ALL1 (MLL) protein, which targets the Drosha-containing microprocessor complex to increase processing of specific miRNA precursors such as miR-191 (Nakamura et al., 2007).

Mouse models overexpressing single miRNA (miR-155) develop lymphoblastic leukemia or high-grade lymphoma, suggesting that deregulation of a single miRNA can cause cancer (Volinia et al., 2006). These cancers become aggressive at slower than usual rates, suggesting that additional events may be required for the cancer to progress at a faster rate. In many lung cancers, the expression of Let-7 family members is either lost or lowered and, as a result, RAS is overexpressed (Johnson et al., 2005). Similarly, loss of miR-15 and miR-16-1 in CLL causes increased expression of Bcl2 and anti-apoptotic factor (Cimmino et al., 2005). The tumor suppressor gene PTEN has been shown to be a target of miR-21 (Meng et al., 2006). In many types of cancer involving various tissues (breast, lung, prostate, etc.), lost or lowered expression of the PTEN gene has been reported, along with increased expression of miR-21. It is important to note that the miR-21-mediated loss of PTEN function has not been observed in cases where a mutation in the PTEN gene occurred. This distinction may have to be made in every tumor where a miRNA–mRNA pair has been implicated to play a role. Questions about these observations still remain. Are there any sub-groups of cells within the tumor mass that show a different miRNA–mRNA expression profile as compared to the rest of the tumor cells? Can this miRNA–mRNA expression profile be used to distinguish cancer stem cells from the rest of the cancer cells within a given tumor?

5.1 miRNA-Mediated Mechanisms in Cancer

5.1.1 Epigenetics and miRNAs

Epigenetic regulations, such as hypermethylation of 5′ regulatory regions of miRNA genes, have been implicated in the down-regulation of miRNA genes in cancers (Lujambio et al., 2007; Saito et al., 2006). Comparisons of the expression levels of miRNAs in normal versus tumor cells found that a sub-group of miRNAs were down-regulated (Calin and Croce, 2006; Chen, 2005; Lu et al., 2005). Since miRNAs are repressor molecules, these down-regulated miRNAs in cancers are termed tumor suppressor miRs. Various studies discovered an intricate link between tumor suppressor miRs and their cognate oncogene pairs (e.g., let-7 and RAS; miR-15/miR-16 and BCL2) (Cimmino et al., 2005; Johnson et al., 2005).

5.1.2 miRNAs in Cancer-Associated Genomic Regions (CAGRs)

Over the years, researchers have identified fragile sites (FRAs) and genomic regions affected in various cancers known as cancer-associated genomic regions

(CAGRs). A close inspection of these regions revealed that many miRNA genes are frequently associated with them (Rossi et al., 2008). Almost half of the human miRNA genes (known at the time of study) have been found near FRAs and CAGRs, suggesting that mechanisms such as deletion and amplification or other genetic modifications could also be important for miRNA-mediated malignancies (Calin et al., 2004). One such region, where the HOX genes or HOX gene family members are clustered along with various miRNA genes, has been shown to be critical for normal development and is frequently altered in cancers (Calin et al., 2004; Cillo et al., 1999; Owens and Hawley, 2002; Pollard and Holland, 2000).

Array comparative genomic hybridization (aCGH) analysis of various cancer samples suggests that copy number abnormality in regions with miRNAs is partly responsible for the progression of cancer (Greshock et al., 2004). Furthermore, these copy number abnormalities have been shown to be associated with expression levels of resident miRNAs in cancer samples, and about 73% of the miRNA genes showed expression reflective of copy numbers (Zhang et al., 2006). Thus, copy number variation may be a critical factor in the abnormal expression pattern of miRNAs in cancers. Simultaneously, a similar alteration in the copy number of genes responsible for miRNA processing and maturation may affect the expression of miRNAs in human cancers. Expression levels of miRNAs have also been shown to be correlated with the cell proliferation index, and miRNA is linked to abnormal cell proliferation, a hallmark of tumorigenesis (Gaur et al., 2007).

5.1.3 miRNA Processing

Precursor miRNA molecules undergo a series of maturation steps, which include processing by Drosha and Dicer and interactions with Argonaute family members (as described in Sect. 2). Any defects due to mutation of genes in these processing factors have been shown to affect normal development and cancer (Carmell et al., 2002; Hutvagner and Zamore, 2002). Often, poorly differentiated non-small cell lung cancer has a lowered expression level of Dicer, and lower Dicer expression/function is a prognostic indicator for surgically treated NSCLC (Karube et al., 2005).

5.2 *Specific miRNAs in Cancer*

MiRNAs seem to regulate various aspects of development and cellular homeostasis, but the mechanistic details for their functions in many aspects of biology remain elusive. Our understanding of miRNA function is limited because multiple miRNAs can regulate a specific mRNA, and a specific miRNA may have multiple targets. There is evidence that miRNA–mRNA pairs can be cell-type or context dependent (Cheng et al., 2005a; Scott et al.,

2007; Shi et al., 2007). Thus, the identification of miRNA-mRNA pairs in a specific cellular context (normal or cancer) is of utmost importance; currently, researchers are using a bioinformatics prediction approach, followed by experimental validation, to gain better insights. However, a systems biology approach may be needed to completely understand the role of miRNA in cancer, as effects of individual miRNAs could be due to the synthetic effects of their targets.

5.2.1 Loss of miRNA Function and Cancer

The miRNAs that function as tumor suppressors (as their loss of function is implicated in tumor pathogenesis) include the let-7 family, miR-15/16, and miR-34. The let-7 miRNA family was first identified in *C. elegans* and was shown to be important for developmental timing (Reinhart et al., 2000). In humans, the let-7 family comprises 11 homologous miRNAs and has been shown to be down-regulated in ovarian, colon, breast, and lung cancers (Akao et al., 2006; Iorio et al., 2005; Park et al., 2007; Takamizawa et al., 2004; Yu et al., 2007). The let-7 family members orchestrate their tumor suppressor functions by targeting known oncogenes and genes known to be involved in cell cycle and cell division control. Oncogenes such as RAS, MYC, and HMGA2 are some of the known targets of the let-7 family of miRNAs (Johnson et al., 2005, 2007; Lee and Dutta, 2007; Mayr et al., 2007; Yu et al., 2007). In ovarian cancers, let-7 may even serve as a potential biomarker (Shell et al., 2007).

The genomic locus for the miR-15a and miR-16-1 cluster happens to be close to 13q14.3, a locus that is often deleted in CLL (Calin et al., 2002). Through gain- and loss-of-function experiments, it was established that miR-15/16 behave as tumor suppressors, blocking the function of Bcl2 (an anti-apoptotic factor) (Cimmino et al., 2005). Expression of these two miRNAs was also shown to be sufficient to induce apoptosis and a concomitant decrease in Bcl2 protein levels in various cancer cells.

Perhaps one of the most important findings in the tumor suppressor field came when it was shown that miR-34 family members possess the two major functions of p53 (namely growth arrest and apoptosis) and are activated by p53. They have been shown to be important in various types of cancers, including colon and lung cancers. Their genomic locations, 1p36 (miR-34a) and 11q23 (miR-34b/c), frequently undergo heterozygous deletions (Calin et al., 2004; Versteeg et al., 1995). In support of these findings, it was shown that the anti-apoptotic protein Bcl2 and many cell cycle regulators such as CDK4, CDK6, cyclin E2, and E2F3 are targets of miR-34 (Bommer et al., 2007).

In many cancers, including breast cancer, gliomas, prostate cancers, and cholangiocarcinomas, miR-21 levels have been shown to be overexpressed, and one of the most prominent targets of miR-21 is PTEN, a negative regulator of PI3K signaling. Multiple functions known to be important for tumor formation and progression, such as proliferation, anti-apoptotic activity, increased mobility, and invasiveness (Meng et al., 2007; Si et al., 2007; Zhu et al., 2008), can be attributed to aberrant miR-21 functions (Chan et al., 2005; Iorio et al., 2005; Meng et al., 2007).

5.2.2 Gain of miRNA Function and Cancer

Various cancers have increased expression of specific miRNAs, such as the miR-17-92 cluster in B-cell lymphomas and miR-155 in lymphomas. The miR-17-92 cluster includes seven miRNAs and is present on the 13q31.3 locus, a locus frequently amplified in B-cell lymphomas. The expression of this cluster is known to be under the control of c-myc. The increased expression of this cluster of miRNAs has been shown to be important for B-cell lymphomagenesis and lung cancer tumorigenesis (Hayashita et al., 2005; He et al., 2005). Tumor growth was further augmented because the miR-17-92 cluster also suppresses the function of the anti-angiogenic factors Tsp1 and CTGF (Dews et al., 2006). Individual members of this cluster, such as miR-20a, have been shown to be oncogenic in prostate cancers, as they act as anti-apoptotic factors. It was shown that mir-17-5p and miR-20a regulate expression of E2F1-3 (Sylvestre et al., 2007). There is a marked increase in the expression of miR-155 in lymphomas, and transgenic models overexpressing miR-155 in B cells have shown B-cell polyclonal proliferation followed by B-cell malignancies (Costinean et al., 2006). These observations established miR-155 as an oncogenic factor. In addition to its role in B-cell lymphomas, miR-155 has been shown to be important in lung, breast, and pancreatic cancers (Gironella et al., 2007; Iorio et al., 2005; Yanaihara et al., 2006). One of the possible targets of miR-155 in B-cell lymphoma is Pu.1, a transcription factor important for early B-cell commitment and development (Vigorito et al., 2007). In pancreatic cancers, miR-155 is known to target the stress-induced cell cycle arrest and pro-apoptotic factor TP53INP1, and their expression levels were inversely related (Gironella et al., 2007).

5.2.3 Gain or Loss of miRNA Function and Cancer

There are groups of mRNAs that can be important in cancer pathogenesis when their expression levels are low (tumor suppressor) or high (oncogenic), depending on their cellular context. This group of miRNAs includes miR-221/222, miR-125b, miR-17-5p, and miR-21. miR-221/222 are located on the X chromosome and have been shown to be up-regulated in many types of cancer, including thyroid cancer, glioblastomas, non-small cell lung cancer, and prostate cancer (Galardi et al., 2007; Garofalo et al., 2008; le Sage et al., 2007; Visone et al., 2007b). The oncogenic functions of miR-221/222 are mediated through down-regulation of $p27^{Kip1}$. These miRNAs function as tumor suppressors in the context of erythroleukemia, where they target mRNA of the proto-oncogene KIT (Felli et al., 2005). The oncogenic function of miR-125b in prostate cancer, where it is shown to facilitate androgen-independent growth, is through targeting of Bak1 (a Bcl2 family member of pro-apoptotic factors) (Shi et al., 2007). In some cases of breast, thyroid, and prostate cancers, miR-125b functions as a tumor suppressor (Ozen et al., 2008; Visone et al., 2007a; Volinia et al., 2006). In breast cancers, it is known to suppress the expression of HER2 and HER3 (Scott et al., 2007). One member of the miR-17-92 cluster, miR-17-5p, is shown to

function as a tumor suppressor in breast cancer cell lines, where it blocks the translation of ACTR/AIB1 (co-activator of p160), thus inhibiting ER and E2F1 signaling (Hossain et al., 2006). In most studies, miR-21 has been shown to be oncogenic; however, in one study in HeLa cervical cancer cells, blocking miR-21 function was shown to significantly increase cancer cell growth, suggesting that in these cells, miR-21 functions as a tumor suppressor (Cheng et al., 2005a).

6 Cancer Stem Cells and miRNA

Although the identity of cancer stem cells is controversial at this time, there is a growing consensus that there are sub-populations of cells in solid tumors that have a higher propensity to give rise to a tumor with features similar to that of the original tumor. These "cancer stem cells" (CSCs) or "tumor-propagating cells" (TPCs) are able to initiate orthotopic tumors in xenograft models, even when they are implanted at very low densities (Reya et al., 2001). These cells have been shown to be resistant to various cancer therapies (chemotherapeutics and radiation) and are capable of recreating the tumor microenvironment, including the vasculature (Bao et al., 2006a,b; Gilbertson and Rich, 2007). Hallmark features of many tumors with poor prognosis, such as elevated cellular migration and establishing themselves in a new niche/microenvironment, can be attributed to CSCs or TPCs. Thus, there is an urgent need to understand the biology of CSCs or TPCs so as to develop effective cancer therapies where the focus will be on treating the minor populations of resistant CSCs or TPCs as well as the bulk of the tumor mass. There are tumors that do not seem to have cancer stem cells, as they are curable through surgery or other treatments (Polyak and Hahn, 2006) and, perhaps, we can identify a special niche or microenvironment (paracrine factors, a specific cellular composition, intrinsic expression profile, or network of factors, including miRNA) in these tumors that inhibits cancer stem cell proliferation or maintenance.

Most of our current understanding of CSCs is derived from studies done in areas such as brain tumors and cancers of the hematopoietic system. Cell types suited for being cancer stem cells include the following: (a) minor self-renewing multipotent quiescent stem cells that acquire mutations and give rise to tumor; (b) somewhat restricted progenitor cells targeted by mutations that subsequently reacquire features of stem cells and give rise to tumor; and (c) terminally differentiated cells that dedifferentiate upon gaining mutations and give rise to tumors (Stiles and Rowitch, 2008). Among all these possibilities, there is evidence to suggest that, most often, the targets of transforming mutations are less mature (differentiated) cells. However, cancer stem cells and tumor cell origin are two independent issues, where CSCs are perhaps the endpoints of the initial tumorigenic transformation events.

It is well documented that cells in tumors experience a variety of stresses, including hypoxia, nutrient deprivation, chemicals, and radiation. It is, thus, very important to look at these factors and their putative role in cancer stem cell

biology. Many cancer stem cells have been found to be more resistant to chemotherapeutic agents and radiation therapies, which points to the unique intrinsic program of these cells. The uniqueness of these cells may stem from the fact that the miRNAs may behave/function differently and thus alter the phenotypic response (Bao et al., 2006a,b; Gilbertson and Rich, 2007). HIFa factors are also known to up-regulate expression of Oct4 (an embryonic stem cell transcription factor) (Covello et al., 2006). Several other factors have been proposed to be regulated by HIFa including activation of Wnt signaling and modulation of Notch activity, MDR1, and ABCG2 (Keith and Simon, 2007). Interestingly, HIFa factors have been shown to induce the expression of various miRNAs and help these cells survive in hypoxic conditions (Kulshreshtha et al., 2007). The functions of miRNA appear to be altered under stress conditions when their binding site on mRNA 3′ UTR is adjacent to RNA binding proteins (Bhattacharyya et al., 2006; Vasudevan and Steitz, 2007); it was observed in these cases that the presence of RNA binding proteins like HuR and fragile X protein FXR1 results in the up-regulation of target genes. It will be interesting to see whether the environmental-stress-mediated alterations in the expression of factors such as Oct4 and miRNAs in cancer cells, and perhaps in cancer stem cells, play a critical role in the establishment and progression of cancers. That stress conditions can not only change the expression patterns but also alter the function of miRNA is fascinating, especially in the context of cancer stem cells.

It is now known that both stem cells and cancer cells (or CSCs?) have somewhat lower overall levels of miRNAs compared to differentiated and normal cells, respectively (Lu et al., 2005; Strauss et al., 2006). The profiles of miRNAs have been useful in grouping various cancers according to their developmental origins and as predictors of therapeutic outcome. There are reports of epigenetic regulation of miRNAs; for example, in human bladder cancer cells, chromatin-modifying drugs led to the up-regulation of a group of miRNAs (Saito et al., 2006).

To date, there have been no reports of miRNA expression patterns in cancer stem cells. Identification of stem-like cells with respect to expression of miRNA (a set of miRNAs or individual miRNAs) within the tumor mass may serve as a useful biomarker. It is not known how critical a role miRNAs play in events such as reacquisition of "stemness" by somewhat restricted progenitors upon transformation or whether the miRNA expression pattern resident to the cell helps or resists such a transformation event. The status of epigenetic machinery in CSCs and what effect it might have on the expression patterns of miRNAs are also unknown.

7 Potential Therapeutic Targets

Small molecules have a distinct appeal as practical and efficient therapeutic agents. In that respect, miRNAs, because they are small in size and are often deregulated in various cancers, have significant therapeutic potential. There are

tools available now that can alter/interfere with miRNA functions in vivo. For example, oncogenic miRNA function can be blocked by using small antisense chemically stabilized RNAs, which can block miRNA inhibitory functions (Li et al., 2006; Lui et al., 2007; Meister et al., 2004a; Weiler et al., 2006). In cancers where loss of miRNA function has been attributed to tumorigenesis, miRNA can be re-expressed by using viral vectors harboring miRNA genes/short hairpin version of miRNAs (Li et al., 2006; Lin et al., 2006). In lung cancers, it has been shown that overexpression of let-7 leads to the decreased resistance of these cells to radiation therapies (Weidhaas et al., 2007). As stated earlier, let-7 expression levels serve as a biomarker to estimate 5-year progression-free survival in breast cancer patients (Shell et al., 2007). It was shown that a ratio of the let-7 target gene HMGA2 to let-7 was useful in prognosis.

Bcl2 is an anti-apoptotic factor commonly deregulated in cancers (e.g., B-cell CLL), with associated loss of miR-15/16. Thus, restoring expression of miR-15/16 in cancers seems a plausible therapy, as they can induce apoptosis by decreasing Bcl2 protein levels. Suppression of miR-221/222 function has been shown in lung cancer models, whereas expression of miR-221/222 in erythroleukemic cells leads to a decrease in the proliferation of these cells. It has been shown that miR-34a is a target of p53 and contributes to normal p53 function. Expression of miR-34a promotes apoptosis and decreased proliferation in various cancers, including lung and colon cancers (Chang et al., 2007; Tazawa et al., 2007). In human gliomas, it has been observed that blockade of miR-21 function in combination with S-TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) leads to a marked decrease in tumor growth (Corsten et al., 2007).

The future of miRNA-centered therapies looks bright. Specific identification of miRNA and its cognate target mRNA is very crucial and is likely to be a key determinant in the success of such therapies. However, the fact that a single miRNA can target multiple mRNAs (and thus affect multiple cellular pathways) remains both a strength and a weakness of such approaches.

8 Conclusions

Our knowledge of miRNA biogenesis and miRNA-dependent regulation of gene expression continues to expand every day, and new pathways are sought for miRNA-dependent control. Knowledge of the regulatory network of miRNA and its targets is proving to be critical for a number of regulatory processes involved in normal development as well as in cancers and other diseases. However, a comprehensive understanding of the critical role of miRNAs in various biological processes and contexts will require additional functional analyses, including a precise identification of miRNA targets. This process has begun in a number of laboratories, but progress will depend on developing more efficient assays for deciphering the targets in different contexts.

Apparently, 30% of all genes and almost every genetic pathway involves regulation by miRNAs (Hwang and Mendell, 2006). As discussed above, miRNAs can function as both oncogenes and tumor suppressors. It will, thus, be very interesting to identify small molecule inhibitors of both the miRNA processing pathway and individual miRNA transcription and function. Until now, specific inhibition of individual miRNA has only been achieved by using antisense molecules (Meister et al., 2004b). Recently, however, for the first time, a specific small molecule inhibitor of miRNA-21 was identified (Gumireddy et al., 2008). The investigators developed a novel method to identify inhibitors of the miRNA pathway in live cells and uncovered Azobenzene 2 from a screening of more than 1000 small organic molecules as a specific and efficient inhibitor of miRNA-21 expression. This opens up a new era of tremendous potential for therapeutics involving misregulated miRNAs in various diseases such as cancer.

From an evolutionary standpoint, a few additional questions remain. How did these present-day miRNAs evolve? Were they present in the prebiotic "soup" 4.5 billion years ago, or did they evolve as complexity in organisms increased? What is the evolutionary pressure for these small non-coding RNAs? These are some of the fascinating questions that should be investigated in the future.

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Cancer Stem Cells and Metastasis: Emerging Themes and Therapeutic Implications

Leah Owens, Benjamin Tiede, and Yibin Kang

Abstract Recent work in the field of cancer research has provided mounting evidence for the role of cancer stem cells in the establishment of many types of tumors. Insights into intrinsic properties of cancer stem cells are especially important in the context of tumor invasion and metastasis. Although more research is still necessary to solidify the role of cancer stem cells in the initiation of metastatic growth, the multistep cascade of tumor growth and progression as it is currently understood encompasses events likely to involve cancer stem cells and the microenvironment that supports them. This chapter will focus on the perceived roles of cancer stem cells in known tumorigenic and metastatic events. Emerging and evolving models of cancer stem cell-mediated tumor progression provide potential windows of opportunity for developing novel therapeutic strategies aiming at thwarting the menacing power of metastatic cancer stem cells.

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1 Introduction

Despite the well-known clinical importance of metastasis and the involvement of secondary site tumors in 90% of cancer-related mortality (Jemal et al. 2007), tumor metastasis is still a mysterious process far from being fully understood (Chambers et al. 2002; Gupta and Massague 2006; Steeg 2006). As early as the late nineteenth century, Steven Paget proposed a classic model of metastasis based on the “Seed and Soil” hypothesis that stressed the importance of metastatic cancer cells and their secondary host organs (Paget 1989). This was followed by the influential hemodynamic (or mechanical) hypothesis proposed by James Ewing that placed the blood flow pattern as a main contributor of organ distribution of cancer metastasis. Today, the era of modern molecular cell biology and genomics has accelerated the discovery of metastasis genes and fueled the debates about the origin of metastatic cancer cells (Hynes 2003; Kang 2005; Weigelt et al. 2005b).

Recent insights into the function and characteristics of cancer stem cells (CSCs) offer a novel approach to understanding the progression of metastasis. Prevailing excitement about CSCs centers on tumor-generating capability in serial transplantation experiments and on the ability of CSCs to mediate chemoresistance. Thus research has focused primarily on the role of CSCs in primary lesions and has lead to discovery that CSCs can drive tumor formation in leukemia as well as various solid tumors. While little work has been done to elucidate the role of CSCs in metastasis, properties of CSCs such as self-renewal and differentiation make them logical candidates for metastatic colonizers. To facilitate the discussion of CSCs with different metastatic ability, a distinction should be made at the beginning of this chapter when referring to two potential subtypes of CSCs. Primary tumor cancer stem cells (pCSCs) constitute the original population of tumorigenic cells which initiate formation of the hematopoietic and solid tumors and are the center of most CSC research. Metastatic cancer stem cells (mCSCs) represent a distinct population of cells with the intrinsic properties to disseminate from the primary site and generate the distant metastases, the leading cause of most cancer mortality.

2 Acquisition of Metastatic Character During Tumor Development

Tumor growth has long been seen as a Darwinian process on a cellular scale. This canonical model involves step-wise mutational alterations which confer a selective advantage and lead to clonal expansion of tumor cells with progressively more pathogenic growth phenotypes (Hanahan and Weinberg 2000). Metastatic traits, while important in invading surrounding stroma, surviving in the circulation, and colonizing foreign tissue, seem to offer little or no selective advantage during early establishment of primary tumors. Thus a puzzle arises as to how a primary tumor acquires metastatic properties (Bernards and

Weinberg 2002). According to the clonal selection model, genomic instability in the progressing tumor leads to multiple heterogeneous populations. Random and sporadic combination of metastasis-enhancing properties in a few rare cells endow them with the ability to break away from the primary tumor mass, migrate to a distant organs, and eventually initiate metastases. Although a possible scenario, this model appears to have difficulty explaining efficient metastatic spread of certain small primary tumors, and the success of the so-called "poor-prognosis signatures," derived from the whole primary tumor population, to predict metastatic risk (Bernards and Weinberg 2002).

Alternatively, mutations endowing metastatic potential could be sustained early in tumorigenesis, giving rise to a tumor in which all cells carry a certain metastatic propensity toward low or high malignancy. Support for early determination of malignancy comes from studies of whole tumor profiling. Risk of metastasis and recurrence can be reliably ascertained by comparing microarray profiles in bulk preparations of primary tumors, suggesting that most cells within the tumor express a similarly predictive gene profile. If genetic properties important to metastatic propensity exist in only a small subset of tumor cells, consensus profiles could most likely not be detected. In breast cancer at least five tumor classes have been identified by gene expression signatures that correspond to varying prognoses for metastasis-free or overall survival. Subgroups identified include luminal A, luminal B, normal breast-like, ERB2-overexpressing, and basal epithelial-like (Perou et al. 1999; Sorlie et al. 2001). Overall poor prognosis based on a panel of factors including metastasis is predicted for basal-like, ERB2+, and luminal B as compared to luminal A and normal breast-like subgroups (Sorlie et al. 2001). Bulk profiling has also been shown to predict prognosis based specifically on metastasis (van't Veer et al. 2002; van de Vijver et al. 2002; Ramaswamy et al. 2003; Wang et al. 2005) and the profiles of distant metastatic cells have been shown to closely resemble that of the primary tumor (Weigelt et al. 2005a). If these profiles could be further subdivided based on specific oncogenic transformation they could become powerful tools both in research connecting metastatic potential to particular mutations and in developing more focused diagnosis and treatment. In fact in mouse models of various breast tumor types, profiling has revealed a generic tumor signature as well as a specific signature dependent upon initial oncogenic events associated with individual prognoses (Desai et al. 2002). While further work is needed to extend this finding to human tumors, it offers further evidence for early establishment of tumor character with regard to metastasis.

Tumor-wide expression analysis still does not address the question as to how mutations are maintained throughout potentially years of tumor growth. One way to reconcile metastatic character and early determination is the idea of adaptation. Metastatic potential could arise from a somatic mutation that allows the cell to respond in a non-cell-autonomous fashion to signals in the microenvironment. For example, later in tumor progression, stroma "activated" by the presence of a tumor (through inflammatory pathways for instance) could provide signals stimulating the epithelial to mesenchymal

transition (EMT) shown to be important in invasion (Scheel et al. 2007). Cells adjacent to this stroma that are primed to respond through an earlier mutation may become preferentially metastatic. More evidence is needed to support this theory, although in pancreatic tumors, EMT is associated specifically with single cells that leave the invasive front closest to the stroma to invade surrounding tissue (Brabletz et al. 2001). CSCs within this population of activated cells would have the potential to invade the circulation and exert their tumorigenic activity in remote organs.

Homogeneity in tumor profiles and association with prognosis cannot exclude the possibility that a small population of highly metastatic cells exist which arose late in tumorigenesis and contribute to malignancy and recurrence. Current microarray profiling techniques are based on total RNA extracts and unless rare metastatic mutants can be sorted from the remainder of the tumor mass, they will not be detected by standard means (Hynes 2003). Regardless of when and how metastatic potential is obtained, understanding the role of pCSCs in tumor growth and metastasis will offer insight into this important question.

3 Tumor Subpopulations, Metastatic Origin, and pCSCs

Despite the obvious importance of bulk profiling in characterizing heterogeneous tumors, it is becoming clear that the majority of cells comprising a tumor actually may arise through propagation and differentiation of a much smaller subset of CSCs. The majority of cancer cells in primary tumors and secondary site tumors lack the capability to initiate new growth as evidenced by the large number of tumor cells found in cancer patient circulation that result in comparatively small numbers (and in some cases complete lack) of metastatic growths (Chambers et al. 2002). In animal models, even when large numbers of tumor cells are implanted, if these cells do not contain a CSC population, no new tumor colonization is detected (Gotzmann et al. 2006). However, if pCSCs are indeed the "germline" of the tumor, giving rise to a body of tumor cells, the question arises as to how these initiating cells are responsible for tumor-wide prognosis signatures which vary greatly between tumor classes. Owing to the range of transformations capable of initiating tumor growth and a myriad of microenvironment interactions, a single pCSC could potentially give rise to various tumor phenotypes depending on oncogenic events sustained early in growth. Some evidence for this model has been shown in leukemia, though more work is needed to elucidate the role of early events in tumor-wide metastatic tendencies. It has been shown that in some leukemias, pre-cancerous CSCs have the ability to propagate both benign and malignant tumor cells depending on microenvironment factors including immune status of the host and site of injection (Chen et al. 2007). Thus it appears that early challenges to even identical pCSCs can alter later tumor phenotype and explain the existence of multiple tumor classes.

While it has been shown that different oncogenic stresses on pCSCs can give rise to tumors of varying malignancy, it is still unclear how these cells are derived. pCSCs could be adult stem cells that acquire cancerous mutations. Alternatively pCSCs may arise from more committed progenitor cells that have regained self-renewal properties along with cancerous phenotypes. These two possibilities are illustrated in Fig. 1. Similar challenges presented to normal stem cells versus their more committed progenitors could result in varying levels of malignancy stemming from the inherent characteristics of the originally targeted cells. Transforming events appear to require the capability of replication which is limited to less-differentiated stem and progenitor cells thereby excluding mature somatic cells as likely CSC precursors. While current prevailing thought favors these more pluripotent cells in formation of CSCs, evidence of cellular plasticity exists that blurs the canonical hierarchy of stem cells and raises questions regarding CSC origins. Terminally differentiated fibroblasts,

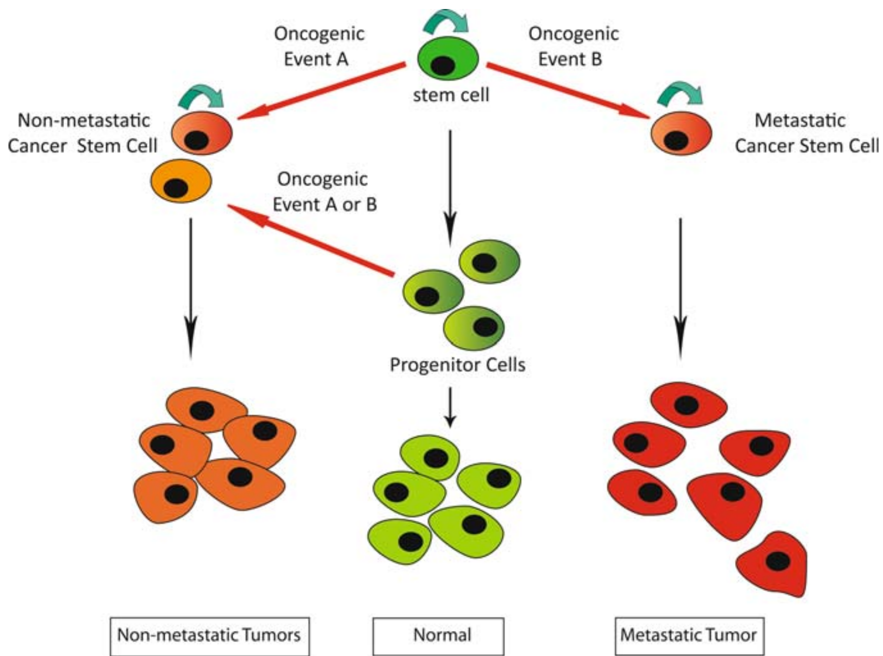


Fig. 1 Models of different CSC origin giving rise to tumors of varying metastatic potential. In normal adult tissues, normal stem cells give rise to more committed progenitor cells which in turn give rise to terminally differentiated cells (*central panel*). Cancer stem cells could arise via oncogenic transformation of either the stem cell population or the progenitor population. Tumors derived from these different precursors and/or with different oncogenic events could show varying malignancy and metastatic potential. Different cellular origin could also explain heterogeneity observed within or amongst tumors. There are speculations that adult stem cells would form the most metastatic lesions owing to their enhanced proliferative capabilities and pluripotency

when manipulated *in vitro* can sometimes take on stem cell qualities of pluripotency and self-renewal (Wernig et al. 2007). Some argue that these are not physiologically relevant phenomena in that they require much cell manipulation, yet they are interesting findings in light of the search for the origins of pCSCs. Though terminally differentiated cell types are unlikely candidates for pCSC formation, studies seeking the sources of pCSCs have found evidence for both stem cells and progenitors (Cairns 2002; Stingl and Caldas 2007).

Normal adult stem cells are likely candidates for pCSC precursors. The most obvious line of evidence for this possibility is that qualities which define a stem cell (self-renewal and differentiation) are also the characteristics that define a CSC. Cancer formation as a step-wise process also requires time for mutations to accumulate. Owing to their inherent self-renewal, stem cells undergo many cell divisions over a long period of time in which multiple DNA-replication mistakes and sporadic mutations can be compounded. Connections between normal and cancer stem cells can also be made through common signaling pathways that have been discovered to mediate function of both cell types. It has been known for many years that Wnt, Notch, Hedgehog, and Bone Morphogenic Protein (BMP) signaling cascades regulate growth and differentiation of normal stem cells. All of these signals have a dual role in that they also affect self-renewal, tumor growth, and malignancy in various cancer models (Piccirillo et al. 2006). Cell surface markers suggest that normal stem cells may give rise to their cancerous counterparts. In hematopoietic cancers, the surface marker signature for normal tissue stem cells $CD34^{+}CD38^{-}Thy1^{-}Lin^{-}$also identifies CSCs (reviewed in Lin and Sessa 2004). It should be mentioned that cell surface marker similarity could arise from progenitor cells re-adopting stem cell markers during tumorigenic growth as has been shown in some normal epithelial cells of the hair follicle (Ito et al. 2007), although few other precedents exist for this phenomenon. Expansion of mammary stem cell populations in mice precede cancer formation, strongly suggesting a role for normal tissue stem cells in the development of malignancies (Shackelton et al. 2006). While this finding has been recently expanded to other cancer model systems (Miyamoto et al. 2000; Kim et al. 2005) there is still no direct connection between adult stem cells and pCSCs. Conclusive evidence would consist of direct transformation of stem cells into pCSCs capable of propagating tumors which may have varying degrees of malignancy. Until researchers are able to better identify and isolate stem cell populations, as has been done in the hematopoietic system, this goal will remain elusive. The difficulty lies in the relative scarcity of stem cells and paucity of reliable in vivo models to isolate them as compared to committed progenitors. Based on their relative abundance, it is possible that committed progenitors cells are more likely to be the population that gives rise to pCSCs.

Since progenitor cells occur typically with much more frequency in adult tissues, statistically they are more likely to sustain the types of oncogenic mutations characteristic of cancer. Strong evidence, especially in leukemias, suggests progenitors as pCSC precursors. In patients with human acute

promyelocytic leukemia (APL) the characteristic chromosome abnormality (a 15:17 translocation) is not found in hematopoietic stem cells; it is found in committed progenitor cells (Turhan et al. 1995). In a mouse leukemia model, myeloid progenitor cells have the ability to induce malignancy if manipulated to overexpress Bcl-2 in conjunction with the translocation-induced oncogene BCR/ABL [B-18], suggesting that early oncogenic manipulation could cause a more committed progenitor cell to gain tumorigenic capabilities. Additionally , the MLL-AF9 fusion protein has been shown to induce granulocyte-macrophage progenitor cells to initiate leukemia in mice (Krivtsov et al. 2006). These same progenitor cells have also been shown to take on self-renewal capabilities via the Wnt/β-catenin signaling pathway (Jamieson et al. 2004), making them candidates for originators of pCSC populations. Granulocyte-macrophage progenitors are particularly interesting in that when transformed by MLL-AF9 they retain a gene expression profile indicative of progenitor cells and do not take on a hematopoietic stem cell signature despite pCSC-like qualities (Krivtsov et al. 2006). Outside of the hematopoietic system, similar evidence is mounting that neural progenitors may give rise to brain tumors (Vescovi et al. 2006) and shows that the role of progenitor cells in oncogenic transformation is likely not merely hematopoietic cancer-specific.

The stronger evidence for progenitors in pCSC formation could simply reflect a bias in experimental design. It is more difficult to find and study adult stem cell populations and the possibility remains that both types of cells could be the source pre-neoplastic cells which sustain further mutation to become fully tumorigenic. Malignant gliomas can be induced from either neural stem cells or transformed astrocytes if the genes for *Arf* and *Ink4a* are inactivated in the presence of EGFR activation (Bachoo et al. 2002). Since these lesions are all highly metastatic, the question remains as to whether or not induction of stem cells versus progenitors can account for tumors of varying metastatic phenotypes. Some precedence for this exists in mammary epithelial cells. Expression of H-ras-v12, hTERT, and SV-40 large T antigen in different mammary cell types results in tumors with different metastatic characteristics, but only when cultured under different conditions (Ince et al. 2007)

pCSC function could also be a more complicated progression involving both types of cells. A stem cell could potentially undergo an early transformative event and give rise to progenitor cells which only then sustains further mutations necessary for malignancy. For example, breast cancer tumor cells expressing the marker $CD44^+$ are closely related to $CD24^+$ cells. $CD24^+$ cells appear to have arisen from $CD44^+$ precursors and sustained further mutations giving rise to a new clonal population. The $CD24^+$cells carry an initial oncogenic transformation shared with $CD44^+$ cells plus additional novel mutations (Shipitsin et al. 2007). Unfortunately, in experiments that investigate a single oncogenic variable, complex interactions such as those seen in breast cancer may not be detectable. For instance perturbing various cell types with specific oncogenic challenges may not reveal the natural step-wise process leading to metastatic cells. Moving beyond analysis of tumor heterogeneity to find the

original pCSC "germline" will be invaluable in understanding pCSCs and utilizing them to improve diagnostics and prediction of metastasis. If, for instance, one could isolate the pCSC population or precursors to them, a more focused gene profile could be used to better determine malignancy and metastatic potential. This profile could then be used to characterize the cellular mechanistic origins of increased metastatic capability.

The need to isolate pCSCs for meaningful analysis of prognosis is evident in breast cancer studies. Markers of pCSCs in solid tumors are still being discovered and debated, but currently the accepted pCSC markers for breast cancer consist of overexpression of the cell surface receptor CD44 and low or absent expression of the anchored membrane protein CD24 (Al-Hajj et al. 2003). It has been shown that characterization of these markers in whole tumors does not correlate with metastatic potential or patient survival (Abraham et al. 2005) highlighting the notion that pCSC may not be equivalent to mCSC. Additionally, some $CD44^+$/$CD24^+$ cells (in one out of nine breast tumors assayed) have been shown to induce tumor formation when injected into mouse recipients (Al-Hajj et al. 2003), implying that these two markers alone may not delineate the pCSC population in all types of breast cancer. Breast cancer being a widely heterogenous cancer type, it will be interesting to see if any markers can accurately identify pCSCs across tumor classes and be used to predict metastatic tendency.

4 Cancer Stem Cells and the Metastatic Cascade

Given that a single cancer cell can drive the formation of a metastatic tumor (Fidler and Talmadge 1986), CSCs are likely responsible for distant tumorigenesis as they are in primary tumor formation. The incongruity between the large number of malignant cells found in the vascular systems of cancer patients and the comparatively small number of macrometastases can be reconciled by the existence of mCSCs. Though other cell subpopulations may break free of the primary tumor and invade the blood stream, mCSCs, like their pCSC counterparts, are those solely responsible for initiation of tumors. mCSCs are related to pCSCs in essential properties of self-renewal and differentiation needed for the propagation of the bulk of the tumor, but differ in key ways. Unlike pCSCs, mCSCs disseminate from the tumor, colonize foreign tissue, and likely have additional alterations (whether mutational, epigenetic, or adaptive) which allow survival and propagation in secondary sites. This has been shown in pancreatic cancer where mCSCs express the same stem cell marker $CD133^+$ as pCSCs, but additionally express a novel CXCR4 marker known to specifically effect migration and homing of metastatic cells (Hermann et al. 2007).

The fact that CSCs can be isolated, not only from the primary tumor, but also from pleural effusions and metastases (Al-Hajj et al. 2003) indicates that these cells have the capacity to migrate and invade other tissue. A common early indicator of metastatic breast cancer is the presence of breast cancer cells in the bones, which in more than 50% of cancers display the $CD44^+$/$CD24^-$

expression profile associated with CSCs (Balic et al. 2006). This strongly suggests existence of mCSCs capable of proliferating in the bone marrow, the most common site of breast cancer metastasis. It should be noted, however, that pioneering studies to identify pCSC markers in breast cancer assayed cells from pleural effusions, not primary tumors (Al-Hajj et al. 2003). It is interesting that the $CD44^{+}/CD24^{-}$ surface marker signature seems to correlate both with pCSC identification as well as with metastatic characteristics. $CD44^{+}/CD24^{-}$ cells exhibit highly invasive phenotypes *in vitro* (Sheridan et al. 2006) and overexpress several genes involved in chemotaxis and motility (Shipitsin et al. 2007). The invasive gene signature found in these cells may be a result of inherent characteristics of the population studied (owing to isolation from outside the primary tumor) or may suggest that pCSC and mCSC populations in some breast cancers are overlapping.

As evident in breast cancer, much work is needed to identify characteristics and origins of mCSCs. Direct confirmation of mCSCs as a unique cell type has only been accomplished very recently in a single pancreatic cancer model (Hermann et al. 2007). A logical approach to this problem would be to use the markers that have identified pCSCs and test them in metastatic assays. Xenograft transplantation of fractionated metastatic tumors or circulating tumor cells could identify mCSC cells by their capability to serially induce tumor formation. This fraction could then be assayed for metastasis-related phenotypes such as ability to colonize various tissues like bone, lung, brain, and other clinically relevant sites. Once a tumorigenic metastatic group is isolated, it can be fractionated based on surface markers and gene expression profiles, yielding a wealth of data critical to the understanding of metastasis. While recent research has focused on endpoints of metastasis measured by expansion of the secondary tumor, other steps of the metastatic cascade must affect mCSCs as well. Processes of angiogenesis, invasion into surrounding stroma, intravasation into lymphatics or blood vessels, adhesion to luminal epithelia, and extravasation should all be explored with regards to mCSC function. This would provide insight into mCSC function and tissue tropisms as well as the process of metastasis in general. Understanding tumor dissemination at a mechanistic level, especially with regards to mCSCs will reveal new potential intervention points for treatment as well as new criteria for diagnosis. Some progress has been made toward this end in angiogenesis induced by mCSCs in glioma. $CD133^{+}$ CSCs are more capable in inducing angiogenesis than their $CD133^{-}$ counterparts (Bao et al. 2006b) suggesting CSC involvement in an early step of metastatic progression and potential for involvement in subsequent steps of the cascade.

5 The Pre-metastatic Microenvironment and mCSCs

Non-cell-autonomous interaction with the environment has long been known to play a key role in cancer metastasis. Signaling interactions with surrounding cells and manipulations of adhesion allowing modified association with the

extracellular matrix are both common changes in cancer cells in the progression toward metastasis. In light of the CSC hypothesis, especially the discovery of specific mCSCs, identifying similar interactions between normal adult stem cells and their microenvironment becomes crucial. Given the close relationship between adult stem cells and CSCs discussed above, it is likely that mCSCs associate with existing cellular niches and utilize them in migration and colonization. A stem cell niche is a supportive microenvironment providing physical attachment and environmental signals important to the growth and regulation of adult stem cells (Schofield 1978). Niches have been extensively characterized in model organisms such as *Caenorhabditis elegans* and in the *Drosophila* germline. In mammals, stem cell niches have been studied in intestine, bone marrow, skin/hair follicles, the neural system, and the testis (Scadden 2006). While these microenvironments differ from tissue to tissue, across types there are conserved themes and signaling pathways important to supporting stem cells. Signal molecules found in niches that regulate normal adult stem cells such as the Wnts and TGFβ are also known to affect tumor growth and metastasis (Li and Neaves 2006). Since the same physical space of the niche could be supportive of both normal stem cells and CSCs, the possibility of competition arises. If the neo-plasticity and oncogenic modifications found in CSCs offer a survival advantage, they could potentially displace normal stem cells to take over the supportive niche. Crowding out normal stem cells could occur during initiation of primary tumors, but could also be important in the establishment of mCSCs in distant organs. In fact, in osteolytic bone metastasis, it has been shown that endosteal osteoclasts become activated (Guise et al. 2006) which can induce the mobilization of hematopoietic progenitor cells within the bone marrow (Kollet et al. 2006).

In breast cancer, where bone marrow is the most common site of metastasis, much evidence points to specific interactions between mCSCs and endogenous stem cell niches. Aside from providing regulation through signaling, niches also serve as anchoring sites for stem cells. Hematopoietic stem cells are attached to osteoblastic niches via N-cadherin/β-catenin complexes (Calvi et al. 2003) regulated upstream by Wnt signaling. The adhesive qualities and population size of HSCs are determined by the glycoprotein Osteopontin (Opn) which is also important in metastasis of breast cancer (Furger et al. 2001; Kang et al. 2003; Stier et al. 2005) implying a role for Opn in mCSC regulation. HSCs also require a calcium-sensing receptor (CaR) to properly localize to bone marrow niches (Adams et al. 2006). Overexpression of CaR in breast cancer tumors correlates strongly with bone metastasis potentially allowing mCSCs to preferentially localize and colonize endosteal stem cell environments (Adams et al. 2006; Mihai et al. 2006).

In addition to taking over defined niches, tumor cells could also manipulate nearby niches to favor growth of oncogenic CSCs. This phenomenon has been observed in basal cell carcinomas (BCCs). Bone Morphogenic Proteins (BMPs) are known to promote stem cell differentiation leading to exit of the stem cell niche and their action is inhibited by the secreted factor Gremlin 1. Tumor cells

derived from BCCs overexpress Gremlin 1 while non-tumor-derived skin cells do not (Sneddon et al. 2006), suggesting that the tumor can influence microenvironmental cues, tipping the balance toward growth of pCSCs and possibly mCSCs. Other interactions between tumor stroma and environment involving immune surveillance and hypoxia pathways have been shown to play a role in metastatic potential (Gupta and Massague 2006; Barnhart and Simon 2007) and may influence the proliferation of mCSCs.

Some of the most exciting developments pertaining to mCSCs regard their ability to condition a pre-metastatic niche in distant tissue prior to tumor dissemination. Tumor tropisms have long been explained by the "Seed and Soil" hypothesis posited by Paget (Paget 1989) in which cancer cells are dispersed "in all directions" but only colonize tissue which happens to provide a supportive microenvironment ideal for growth. Recent functional genomics studies have at least partially validated this model by demonstrating subpopulations of tumor cells with tissue-specific metastatic tendencies (Kang 2005; Minn et al. 2005a). On the other hand, gene signatures identified through this approach could also be used to predict organ-specific metastasis tendency based on the overall primary tumor gene expression profile (Minn et al. 2005b). Explanation for these preprogrammed tendencies came in a groundbreaking report in 2005 showing lung and melanoma cells recruit factors to future metastatic sites (Kaplan et al. 2005). Secretion of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) by primary tumor cells recruits bone marrow-derived cells (BMDCs) to what is now referred to as the pre-metastatic niche. Interfering with BMDC induction by blocking secretion of these factors virtually eliminates metastasis in these cancers. Injecting animal hosts with media conditioned by tumor cells was sufficient to re-direct tumor tropism to the organ associated with the source of secreted factors. The mechanism of this effect is still unclear, but potential candidates for involvement are the cytokines S100A8 and S100A9 which are expressed by myeloid cells and upregulated in lungs of pre-metastatic tumor-bearing mice. While untreated animals develop metastases, inhibiting the overexpression of these cytokines in lung tissue greatly reduces metastasis to lung tissue. Induction of the p38 MAP kinase pathway involved in tumor cell migration and invasion likely helps mediate this cytokine response (Hiratsuka et al. 2006). Activation signals may also be required for metastatic growth even after mCSCs reach the secondary site. It is thought that mCSCs, for instance in breast cancer, can migrate to bone marrow or lung tissue and enter a quiescent state (Li et al. 2006) thus requiring an activation signal to induce growth of macrometastatic lesions. While there is little evidence for some steps of the mCSC-mediated metastatic cascade (such as reactivation), the overall model provides a working framework of mCSC function as summarized in Fig. 2. This model highlights new potential interactions of mCSCs with the metastatic niche and illuminates novel therapeutic targets to block these interactions.

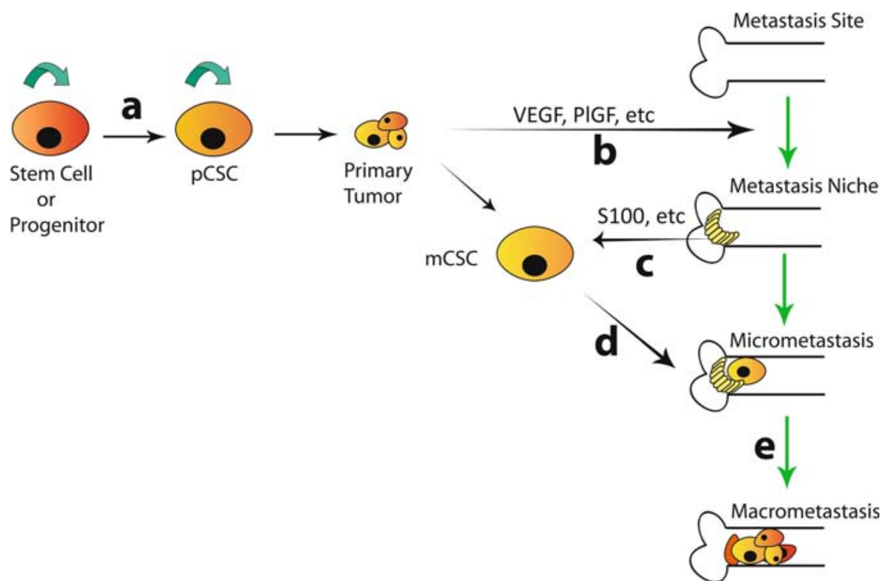


Fig. 2 Model of the mCSC-mediated metastatic cascade. An initial oncogenic event gives rise to a pCSC capable of proliferating to induce a tumor (**a**). Depending on the inherent metastatic characteristics of the pCSCs, signals such as VEGF and PlGF are secreted into the systemic circulation to prime and remodel pre-metastatic niches (**b**). Factors released from the pre-metastasis niche can signal back to tumor cells to promoter invasion and metastasis (**c**). mCSCs migrate into the circulation and home in on target organs with appropriate niches (**d**). Once anchored to the secondary site the mCSC may enter a quiescent state (micrometastasis) until activating signals in the environment induce it to begin proliferation and drive formation of a metastatic tumor (**e**)

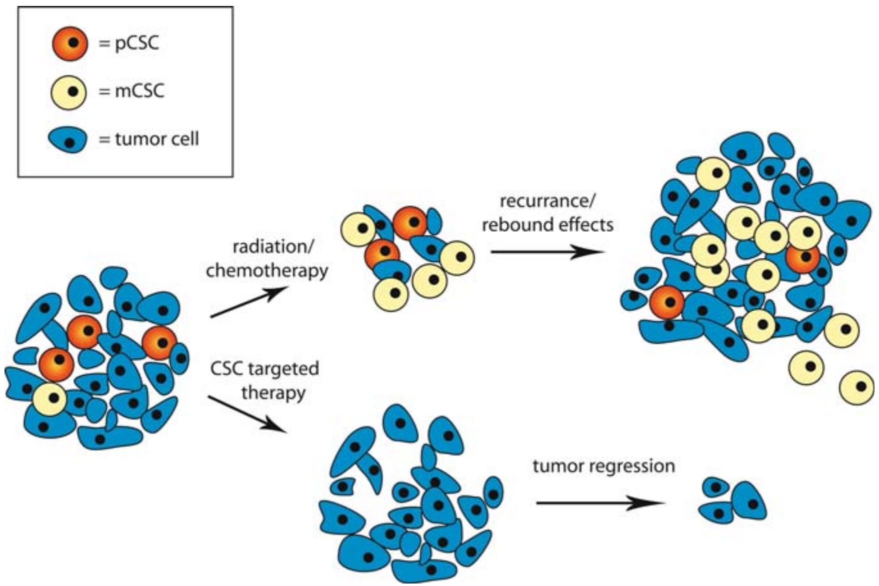
6 mCSCs: Novel Opportunities for Therapeutic Intervention

Understanding the role of mCSCs in cancer progression and fatality will open many new opportunities for clinical intervention which may better target the source of metastatic growth. If CSCs truly are the root of malignancy and solely responsible for tumor growth, only therapies that deplete this population will ultimately be successful as an effective cure. A greater understanding of CSCs is still needed to develop therapeutics directly targeting them, but progress is being made in this direction.

Current standard treatment for most cancers involves some combination of chemotherapy, hormonal therapy, radiation treatment, as well as a growing list of molecular targeting therapeutics, depending on tumor characteristics and stage. Following treatment, tumor regression is normally used as an indicator of therapeutic success. In order to better treat cancer, new ideas about CSCs must be integrated into our understanding of clinical intervention. Both standard treatment and current diagnostic end points are likely not applicable to the CSC

model. Overall tumor regression based on size offers no indication that the rare pCSCs responsible for primary lesion growth or mCSCs responsible for metastasis have been depleted. A better therapeutic endpoint would involve direct sampling of CSC populations (which would require better markers for fractioning cancer cell populations).

Unfortunately standard treatments have been shown to have little or no positive effect on CSCs (Hermann et al. 2007; Ho et al. 2007; Hermann et al. 2008; Ma et al. 2008). In pancreatic tumor cells, treatment with the first line chemotherapeutic agent Gemcitabine has no effect on the $CD133^+$ population thought to encompass pCSCs and in mouse models actually leads to enrichment for this population (Hermann et al. 2008) . CSCs are also resistant to therapeutic levels of radiation in human glioma tumors and treatment causes increased tumor aggressiveness in some cases (Bao et al. 2006a). The $CD44^+$ population in breast cancer also appears to be radio-resistant (Phillips et al. 2006). The rebound effect seen in all of these models where treated tumors relapse with greater malignancy could be explained by the fact that chemotherapy and radiation decrease tumor bulk without affecting CSCs (as shown in Fig. 3), thereby enriching for the tumor-initiating cells. This response could be attributed to enhanced DNA repair, anti-apoptotic protein expression, or the presence of drug-efflux pumps on CSC membranes, all of which have been suggested in various tumor types (Sleeman and Cremers 2007). Furthermore, a new class of dual-functional genes that contribute to both chemoresistance and metastasis (Hu et al. 2007) can be enriched in mCSC after therapeutic exposure. Understanding the metastatic process reveals several points at which



therapeutic intervention may be applied to specifically target and deplete mCSCs leading to greater efficacy in treatment. Summarized below are several such points and potential approaches based on what is currently known about them.

6.1 Targeting the (Pre-)metastasis Niche

Tissue-specific tropisms and interactions of mCSCs can be exploited with appropriate therapeutics. The metastatic microenvironment affects homing, adhesion, and proper functions of mCSCs, including self-renewal and differentiation, any of which could prove useful pathways to target at the molecular level using inhibitors or competitors of key factors. After it was shown that secretion of VEGF and PlGF initiated remodeling of pre-metastatic niches, it was also shown that inhibiting these factors with specific antibodies reduced metastasis (Kaplan et al. 2005). It has been known for some time that VEGF is an angiogenic factor associated with malignancy. The possibility that the underlying mechanism involves mCSCs is still debated (Jain 2001; Lin and Sessa 2004). Nonetheless a VEGF antibody drug (bevacizumab) is currently in clinical trials based on its anti-angiogenic properties (Hurwitz et al. 2004). It will be an important step in understanding mCSCs to see if it can increase survival in patients with recurrent tumors that have proven refractory to standard treatment.

As niches provide anchor sites for mCSCs this adhesion could also be blocked. CD44 surface receptor inhibition leads to reduction of engraftment of cancer cells in acute myelogenous leukemia when injected directly into bone marrow (Jin et al. 2006). CD44 has also shown similar effects in chronic myelogenous leukemia (Krause et al. 2006) and is important as a marker of

Fig. 3 Traditional and CSC-based approaches in cancer treatment. Chemotherapy and radiation are standard treatments for tumors but do not specifically deplete CSCs. A tumor comprises a small subset of tumorigenic, self-renewing CSCs and a bulk of cancer cells incapable of propagating new tumor growth. mCSCs break away from the malignant tumor to cause metastasis. Use of non-specific radiation or chemotherapy may shrink the tumor but does not deplete more chemoresistant CSCs. Over time CSCs repopulate the tumor through proliferation and may be more aggressive/invasive than the original tumor (referred to as rebound effects) as radiation/chemotherapy may select for mCSCs due to the coupled activity of metastasis and chemoresistance encoded in certain dual-functional genes. mCSCs proceed to colonize secondary tissue and produce recurrent metastasis (*upper panel*). Therapies that specifically target and deplete CSCs may not have a dramatic effect on tumor size by current standards, but do abrogate long-term tumorigenic potential. Over time the bulk of the tumor shrinks as tumor cells are eliminated through natural attrition or conventional chemotherapies and are not replaced. New drugs are needed to accomplish such targeted therapy and new clinical assays are also essential for measuring the success of these novel therapies

CSCs in breast cancer metastasis. Further exploration of CD44 inhibition may result in powerful anti-metastatic drugs in multiple cancer types. Another potential target is CXCR4 which is important in cancer metastasis of breast, prostate, and other cancers (Muller et al. 2001; Taichman et al. 2002; Kang et al. 2003), in pancreatic mCSC metastasis (Hermann et al. 2007), and in adhesion of hematopoietic tumor cells to their putative niche (Tavor et al. 2004). Metastatic niche signaling pathways that promote proliferation and downregulate differentiation will also be interesting targets for molecular intervention. Further characterization of these interactions is needed to specifically block metastatic growth without adversely affecting large numbers of adult stem cells which rely on the same signaling molecules for normal function.

6.2 Transition of Quiescence (Micrometastasis) to Proliferation (Macrometastasis)

It is theorized that mCSCs disseminate from the primary tumor, potentially in the early stages of growth, and lodge in secondary sites where they can remain dormant. Latency between migration and metastatic growth could be determined by induction signals in the pre-metastatic niche. While this process has yet to be confirmed, reactivation signals would be an ideal target for drug interference. By the time a tumor is detected it is likely that mCSCs have already invaded and adhered to distant organs, reducing the efficacy of drugs interfering with mCSC migration or adhesion. Currently reactivation of mCSCs remains a theory and effective drugs remain far in the future. Nevertheless it remains an exciting possibility and an area for innovative research.

6.3 Targeting CSC Surface Markers

Cell surface markers (such as CD133 in pancreatic tumors and CD44 in breast cancer) generally used to identify mCSCs could also be used to target these cells for clearance from the body. DNA vaccines and oncolytic virus approaches could be useful in selectively killing these most dangerous cells. Some evidence for potential in this direction has been shown in breast cancer pleural effusions. When $CD44^{+}/CD24^{-}$ cells are infected with adenoviruses with modified capsid protein they lose their ability to generate tumors (Eriksson et al. 2007). The same viruses can deplete the population of $CD44^{+}/CD24^{-}$ CSCs in established tumors. As opposed to non-specific chemotherapy or radiation, this type of treatment could decrease the risk of recurrence and rebound effects. Caveats to this approach include incomplete understanding of marker specificity and the fact that normal stem cell populations often share surface markers with CSCs. Further research is essential to finding ways to exploit surface markers without depleting systemic levels of adult stem cells.

7 Conclusion

Over the last several years, the cancer stem cell theory has gone from mere speculation to a highly evidenced working model of tumorigenesis. Regardless of whether these CSCs come from adult stem cells or progenitors, it is clear that they have a role in initial tumor formation and in metastasis. Understanding CSCs on a molecular, tissue, and organism level is crucial to understanding the true nature of cancer. Starting with better diagnostics based on CSC markers and eventually targeting drugs to pCSCs and mCSCs will undoubtedly result in improved prevention and treatment of many types of cancer.

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Stem Cells in Leukemia and Other Hematological Malignancies

Mhairi Copland, Alison M. Michie, and Tessa L. Holyoake

Abstract Leukemia was the first malignant condition in which cancer stem cells were described. Leukemia stem cells (LSCs) have now been described in a number of different types of leukemia and are currently a major focus of research interest. LSCs are an important target for treatment of leukemia, and failure to eradicate these very primitive cancer cells is a common cause of leukemia relapse. Therefore, improved understanding of the biology of LSCs and the differences between normal hematopoietic and leukemic stem cells is likely to lead to the development of novel therapeutic strategies and improvements in leukemia therapy and patient survival.

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1 Hematopoietic Stem Cells and Normal Hematopoiesis

Stem cells are defined as cells that can differentiate into multiple different cell types and have the ability to self-renew. There are two broad categories of stem cells: (1) the pluripotent stem cell which can differentiate into endoderm, mesoderm, and ectoderm, e.g., embryonic stem cells and (2) multipotent stem cells which are lineage-specific and include hematopoietic stem cells (HSCs). The HSC is a relatively rare cell within the bone marrow, and it is estimated that there are between 3×10^5 and 4×10^6 HSC in the human, based on studies using limiting dilution analysis in non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice (Wang et al. 1997) and long-term culture-initiating cell (LTC-IC) assays (Pettengell et al. 1994). Further studies indicate that each HSC divides approximately 70 times during its lifetime (Vickers et al. 2000) so its self-renewal capacity is finite. By this process of cellular amplification, it is estimated that if one stem cell divides 20 times then 1×10^6 mature cells are produced.

Hematopoiesis is the process of blood cell production. As the majority of mature blood cells only live for a short time in the circulation (a few hours in the case of granulocytes) before destruction by the spleen, it is necessary for the bone marrow to produce up to 10^{13} cells per day to maintain the hematopoietic system. The process of hematopoiesis begins with the multipotent HSC which has self-renewal capacity and the ability to differentiate into all types of mature blood cell (myeloid, erythroid, lymphoid, etc.) through a range of lineage-committed progenitor cells (Fig. 1).

The ability of HSCs to self-renew is heterogeneous and studies on mouse bone marrow cells indicate that 0.05% of bone marrow cells are multipotent progenitors. This HSC population can be divided into three distinct maturational subpopulations: long-term self-renewing HSCs that produce mature hematopoietic cells for the lifetime of the mouse; short-term self-renewing HSCs; and multipotent progenitors which have lost the ability to self-renew, reconstitute lethally irradiated mice for less than 8 weeks and have increased mitotic activity (Morrison et al. 1997).

It is believed that, in steady state, only a minority of HSCs reconstitute the hematopoietic system, with the vast majority of HSCs existing in a quiescent state (i.e., in G_0; out of the cell cycle). This extended period in G_0 allows the resting HSCs time to repair any DNA damage and maintain their genetic integrity (Lajtha 1979). Evidence for the existence of HSCs in a quiescent state came from culture studies in which primitive human progenitor cells remained as single cells for as long as 2 weeks and only began proliferation after stimulation with a cytokine cocktail (Leary et al. 1989, 1992).

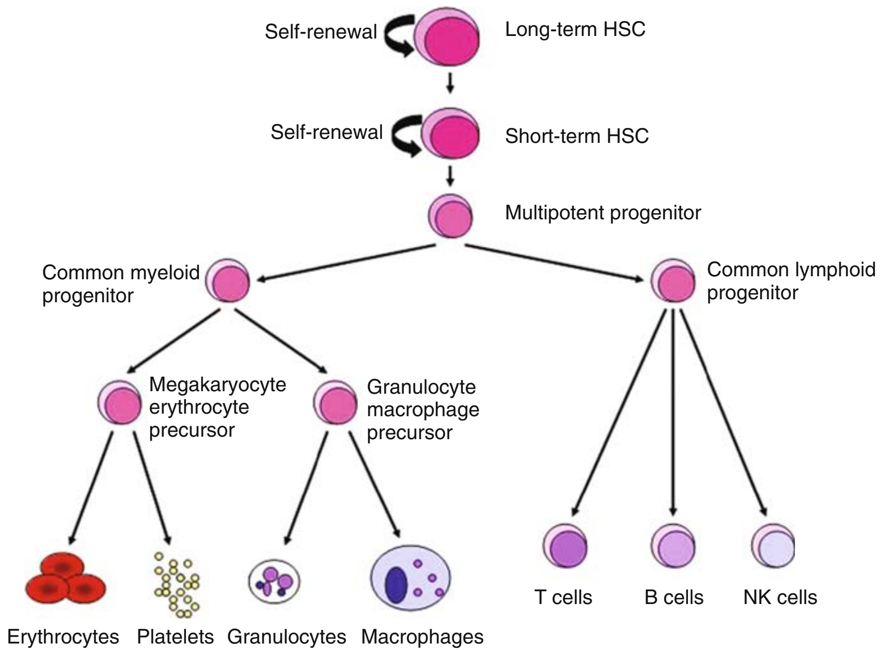

Fig. 1 Diagrammatic representation of the HSC and the cell lineages produced from it. Only long-term and short-term stem cells undergo self-renewal. The multipotent progenitors do not undergo self-renewal, but give rise to common myeloid and common lymphoid progenitors, which through several proliferation and differentiation steps, give rise to the mature cells of the hematopoietic system

Candidate human HSCs are characterized as $CD34^{+}lin^{-}CD38^{-}$ (Miller et al. 1999) and these cells have the ability to repopulate SCID mice. However, for clinical and the majority of research purposes, HSCs tend to be isolated on the basis of CD34 expression only, resulting in a heterogeneous population of which only a small proportion are multipotent HSCs. The CD34 antigen is a transmembrane glycoprotein and member of the sialomucin family (Simmons et al. 2001). Although its precise function is unknown, CD34 is believed to be involved in cell adhesion.

1.1 Early Evidence for the Existence of HSCs

In the 1950s, early studies of murine hematopoiesis, using lethally irradiated recipient mice and marker chromosomes to separate host and donor cells demonstrated reconstitution of the entire hematopoietic system from bone marrow cells (Ford et al. 1956). However, it was not until 1961 that Till and McCulloch provided evidence for the existence of multipotent HSCs (Till and

McCulloch 1961). In their seminal publication, again using marker chromosomes, they demonstrated that multi-lineage splenic colonies arising from a single cell were formed after the injection of bone marrow into irradiated murine recipients. Furthermore, these single cells (termed the spleen colony-forming unit [CFU-S]) were capable of producing new colonies in secondary recipients, demonstrating self-renewal capacity (Siminovitch et al. 1963). However, subsequent studies demonstrated that CFU-S were a heterogeneous cell population and incapable of producing lymphocytes and thus, could not be considered true pluripotent HSCs (reviewed by Iscove 1990). More recently, the surface phenotype of pluripotent HSCs was described following advances in cell purification techniques including fluorescence-activated cell sorting (FACS; Spangrude et al. 1988) and the development of efficient retroviral gene transfer techniques which permit clonal marking of the progeny of individual HSCs (Guenechea et al. 2001).

1.2 Techniques for Studying Stem Cells in the Hematopoietic System

The in vitro study of HSCs is difficult for a number of reasons. First, these are rare cells with only relatively small numbers present in any individual. Second, in vitro culture results in varying degrees of expansion and differentiation of these cells depending on the culture conditions. This results in the loss of stem cell phenotype as the cells mature and acquire lineage-specific markers, and the stem cells become diluted by their more mature progeny. Third, despite extensive studies into the functional and phenotypic properties of HSCs (Weissman 2000), the mechanisms which control self-renewal versus proliferation and differentiation remain unexplained. The HSC achieves both these functions via asymmetric cell division in which one new HSC is produced along with one daughter which then undergoes symmetrical division and differentiates. However, HSCs can also undergo symmetrical cell division to produce two daughter cells. It is believed that stem cell fate is decided by, as yet unidentified, factors in the stem cell niche (Wilson and Trumpp 2006).

Several combinations of cell surface markers have been used to identify and/or purify murine and human HSC by FACS. Murine HSC have been identified as Lin^{-}, $Thy1.1^{lo}$, $Sca\text{-}1^{+}$, $c\text{-}Kit^{high}$, $rhodamine123^{lo}$, and $CD34^{-/int}$ and human HSC as Lin^{-}, $Thy1^{+}$, $CD34^{+}$, and $CD38^{neg/lo}$ (Passegue et al. 2003). However, all cells with these phenotypes are not true HSC; in murine bone marrow, the frequency of true HSC is ~0.02%, making the HSC a very rare cell indeed. Very recently, in human cord blood, improved purification of HSC was achieved by isolating low-density lineage-negative or $CD34^{+}$ cells with elevated levels of aldehyde dehydrogenase activity (Christ et al. 2007). This gave a much improved long-term repopulating cell frequency of 0.3%. In further studies to characterize the murine HSC compartment, Kiel et al. compared the gene

expression profiles of highly purified HSCs and non-self-renewing multipotent progenitor cells (Kiel et al. 2005). Cell surface receptors of the Signaling Lymphocyte Activation Molecule (SLAM) family, including CD150, CD244, and CD48 were differentially expressed between different primitive progenitor subpopulations. HSCs were $CD150^{+}CD244^{-}CD48^{-}$, whereas multipotent progenitors were $CD150^{-}CD244^{+}CD48^{-}$ and lineage-restricted progenitors were $CD150^{-}CD244^{+}CD48^{+}$. This was the first description of a receptor family whose expression combination distinguished HSCs from progenitor cells.

Several models have been developed to study HSC both in vitro and in vivo. Primitive hematopoietic cells with proliferative potential can be maintained in culture for extended periods of time, typically several months. These culture conditions have been called long-term bone marrow culture (LTBMC; Coulombel et al. 1983). Briefly, LTBMC requires the formation of a supportive stromal layer which supplies the necessary microenvironment to allow the primitive hematopoietic cells to proliferate over time. An application of LTBMC is an assay that measures the number of LTC-IC (Sutherland et al. 1991; Hogge et al. 1996) and is the most stringent in vitro stem cell assay. In this assay, the cells of interest are overlayed on pre-established, irradiated stromal layers. After 5–8 weeks culture the contents of each plate are set up in a committed progenitor assay called a colony-forming unit (CFU) assay for a further 2 weeks. At the end of this time, the number of colonies formed is counted and this allows the frequency of LTC-IC to be determined.

Models using SCID (lack B and T cells) and NOD-SCID (lack B, T, and NK cells and have other immune deficiencies) mice have been extensively used to study normal HSC and leukemic stem cells (LSCs) (Holyoake et al. 1999a,b). The transplantation of HSC into SCID or NOD-SCID murine models and reconstitution of hematopoiesis are used to define HSCs as SCID-repopulating cells (SRC). In addition, SCID and NOD-SCID models have also been used to identify SCID leukemia-initiating cells (SL-IC), which are human leukemia progenitor cells with the ability to reconstitute leukemia in these murine xenotransplantation assays. In SCID or NOD-SCID models, following sublethal total body irradiation, normal or leukemic human cells are injected intravenously into the mice. Six weeks to 6 months later, the mice are sacrificed and the bone marrow is harvested and stained with human-specific antibodies to identify the engraftment of human hematopoietic cells by FACS (Holyoake et al. 1999a). NOD-SCID mice are superior to SCID mice for functional assessment of HSC and LSC as, in SCID models, large numbers of cells need to be inoculated into the mice for engraftment to occur and serial transplantation is not possible. Serial transplantation experiments in NOD-SCID mice have now become the method of choice in many laboratories for assessing the engraftment potential and self-renewal capacity of normal and malignant HSC.

Limiting dilution analysis is widely used both in vitro and in vivo to enumerate LTC-IC or HSC and provide information on an individual cell's potential (Coulombel 2004). This technique uses varying dilutions of cell suspensions

which are either plated out in vitro or inoculated in vivo (Sieburg et al. 2002). In LTC-IC experiments, at the end of the culture period, the proportion of positive wells (contain at least 1 CFU) is determined and plotted against the number of input cells to the LTC-IC assay. The Poisson statistic is then used to assess the frequency of LTC-IC in the input cell culture, and the absolute number of LTC-IC and mean CFU per LTC-IC are calculated. Limiting dilution analysis is used in a similar manner in murine in vivo HSC models to determine the frequency of SRC and SL-IC (Wang et al. 1997).

2 Leukemia Stem Cells

2.1 Myeloid Leukemias

Following the identification and characterization of the HSC, comparisons were drawn with the behavior of cancer cells, in particular leukemias, with the first evidence for the 'cancer stem cell' being described in acute myeloid leukemia (AML) (Blair et al. 1997; Bonnet and Dick 1997). Bonnet and Dick demonstrated that the SL-IC, which was capable of causing human AML in NOD-SCID mice, possessed the potential for self-renewal and capacity for differentiation and proliferation predicted for a LSC. Furthermore, the SL-ICs from all subsets of AML, regardless of morphological heterogeneity in maturation of the leukemic blasts, were exclusively $CD34^{+}38^{-}$, analogous to normal SRC. This suggested that normal primitive progenitor cells and not committed progenitor cells were the target for leukemic transformation. Importantly, SL-ICs were capable of differentiating in vivo into leukemic blasts, providing the first evidence that the leukemic clone, like normal hematopoiesis, was organized as a hierarchy. Further support for this hypothesis came from Hope et al. (2004), who were able to track individual human LSCs, using NOD-SCID mice serially transplanted with human AML cells. They showed that, like normal hematopoiesis, the LSC compartment comprised a hierarchy with distinct LSC fates decided by heterogeneous self-renewal potential and that normal development pathways were not entirely eradicated by leukemogenesis.

The cell surface phenotype of LSCs was further elucidated by Blair et al., who demonstrated that, at diagnosis, the majority of AML blasts lacked expression of Thy-1 (CD90), CD71, HLA-DR, and CD117 which differentiated primitive AML progenitor cells from normal hematopoietic progenitor cells (Blair et al. 1997, 1998; Blair and Sutherland 2000), and Jordan et al. who showed that the interleukin-3 receptor α chain (IL-3α) is a unique marker for human AML stem cells (Jordan et al. 2000). Very recently, CD96 has been identified as a surface marker on the majority of $CD34^{+}38^{-}$ AML cells, with minimal expression on normal $CD34^{+}38^{-}$ cells (Hosen et al. 2007). Furthermore, in murine transplantation experiments, only $CD96^{+}CD34^{+}38^{-}$ cells showed significant bone marrow engraftment in recipient mice, indicating

that CD96 is present on the majority of AML LSC and may be useful in developing LSC-specific therapy in the future.

Thus, it is now believed that the LSC is derived from an HSC following one or more leukemogenic events (Fig. 2). These cells, also called SL-IC, make up a very small proportion of the total leukemic cell population (0.2–1%). Chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) have also been described as HSC disorders (Eaves et al. 1998; Cobaleda et al. 2000).

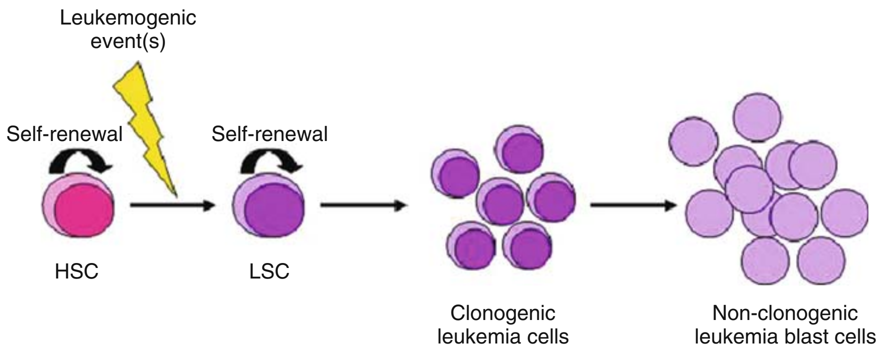

Fig. 2 Schematic diagram of the transformation of an HSC to a LSC which also retains the capacity to self-renew. Following a number of intermediate progenitor stages, the leukemic blast cells are produced and these blast cells form the vast majority of leukemic cells present

CML is a clonal myeloproliferative disorder which develops when a single, multipotent HSC acquires the Philadelphia (Ph) chromosome, which is an abnormal, shortened chromosome 22 that results from a reciprocal translocation between the long arms of chromosomes 9 and 22 and is designated t(9;22)(q34;q11) (Rowley 1973). In the 1980s, it was shown that this translocation resulted in the *ABL* proto-oncogene, normally on chromosome 9, becoming juxtaposed with the *breakpoint cluster region* (*BCR*) on chromosome 22 (Bartram et al. 1983; Groffen et al. 1984), resulting in production of the unique fusion gene product BCR-ABL, a 210 kD oncoprotein, often referred to as $p210^{BCR\text{-}ABL}$, which is a constitutively active tyrosine kinase (Lugo et al. 1990) and results in increased proliferation, abnormal cell adhesion, and reduced apoptosis of the malignant clone.

CML progresses through three phases, the first is chronic phase which is associated with expansion of the granulocyte series with the cells retaining the capacity to differentiate. In the next phase, accelerated phase, there is the development of additional cytogenetic abnormalities and increased numbers of more primitive cells in the peripheral blood. The final phase, blast crisis, is characterized by the expansion of myeloid or lymphoid blasts, with loss of the ability of these cells to differentiate, and this final phase behaves like an acute

leukemia. Interestingly, the Ph chromosome is also found in ~20% of de novo adult ALL and <5% of pediatric ALL and, if present, carries a very poor prognosis (Wong and Witte 2004). The first evidence for the presence of LSC in CML came in 1998 when Wang et al. demonstrated that sublethally irradiated NOD-SCID mice, intravenously injected with peripheral blood or bone marrow from CML patients, had persistence of normal and leukemic human cells in bone marrow for up to 7 months (Wang et al. 1998). Additional research has shown that, in CML, *BCR-ABL* can be detected in myeloid, megakaryocytic, erythroid, and B-lymphoid lineages, accounting for the transformation to an acute myeloid or lymphoid leukemia when the disease progresses into blast crisis (Fialkow et al. 1977; Martin et al. 1980). This provides further evidence for the candidate LSC in CML being an HSC. However, a more recent study by Jamieson et al. indicates that, in blast crisis CML, granulocyte-macrophage progenitors acquire the potential for self-renewal through activation of β-catenin (Jamieson et al. 2004). This acquisition of stem cell characteristics by a more mature progenitor population is postulated to be responsible for the transformation of CML from chronic or accelerated phase to blast crisis.

The identification of LSC in myeloid leukemias has served as an important model system for advancing the study of cancer development in both leukemias and solid tumors. At present, the hypothesis for the development of myeloid leukemias is that the less aggressive myelodysplastic syndromes (MDS) and myeloproliferative disorders progressively evolve into acute leukemias which are rapidly fatal if left untreated. Numerous different cytogenetic abnormalities have been described in association with myeloid leukemias (McCormack et al. 2008), with specific abnormalities being associated with specific subtypes of leukemia with different laboratory and clinical features.

In most leukemias, although the blast cell population is morphologically homogenous, the leukemic cells are functionally heterogeneous. At any time, like HSC, the majority of LSC are quiescent (in G_0). This was first demonstrated in a SCID murine model in which mice transplanted with primary leukemia cells had residual leukemia following treatment with the cell cycle-specific agent 5-fluorouracil (Terpstra et al. 1996). CML is incurable using repeated AML-type chemotherapy (Kantarjian et al. 1991) suggesting that there is a pool of quiescent CML stem cells which are resistant to treatment. Studies have now confirmed the existence of a highly quiescent population of LSC in CML (Holyoake et al. 1999a). In this study by Holyoake et al., viable BCR-ABL$^+$ G_0 cells were isolated using Hoescht 33342 and Pyronin Y staining from total CD34$^+$ CML cells by FACS and were demonstrated to have in vitro progenitor activity by LTC-IC assay and the capability of engrafting immunodeficient mice. The ability of these quiescent CML stem cells to produce leukemic progeny also illustrates the reversibility of this quiescent state. Further research showed that the entry of BCR-ABL$^+$ progenitors into a quiescent state in vivo was greatest in the most primitive leukemia cell populations. This was associated with downregulation of IL-3 and G-CSF gene expression, and spontaneously reversed in association with upregulation

of IL-3 expression and entry of cells into a continuously cycling state (Holyoake et al. 2001). The phenomenon of quiescence in LSC is important because it would be predicted that the growth factor-independent quiescent LSC would have a proliferative advantage over normal quiescent HSCs when the concentration of cytokines is low.

2.2 Acute Lymphoblastic Leukemia

Cobaleda et al. demonstrated that, in NOD-SCID mice, the SL-IC capable of inducing human Ph^+ALL possessed the differentiation and self-renewal capacity of a candidate LSC (Cobaleda et al. 2000). In these studies, the SL-ICs from all animals analyzed were exclusively $CD34^+CD38^-$, with a similar cell surface phenotype to SRC, indicating that an HSC and not a committed progenitor is the target for leukemic transformation in Ph^+ALL. However, the true identity of the LSC in ALL has been controversial. Cox et al., again using a NOD-SCID model, demonstrated that cells with a more immature phenotype ($CD34^+$ $CD10^-CD19^-$), rather than committed B-lymphoid progenitors were the most likely candidate LSCs for transformation to B-ALL (Cox et al. 2004). In addition, this study provided evidence for a hierarchical organization of ALL progenitors as the majority of $CD45^+$ cells harvested from the NOD-SCID bone marrow were $CD34^+$, $CD10^+$, $CD19^+$, and $CD22^+$, indicating that $CD34^+CD10^-$ and $CD34^+CD19^-$ subpopulations of B-ALL cells underwent a degree of differentiation in vivo. Contrary to this, other studies have identified the ALL-propagating cells as $CD19^+$. Castor et al. demonstrated that while in p210 Ph^+ALL the candidate LSC was an HSC, in other subtypes of ALL (p190 Ph^+ALL and TEL-AML1 fusion) the cell targeted for subsequent leukemic transformation was a committed B-cell progenitor (Castor et al. 2005). Very recently, Hong et al. have studied a monochorionic twin pair with the TEL-AML1 fusion, one with frank leukemia and one in the pre-leukemic phase (Hong et al. 2008). This important research using primary human material has shown that a $CD34^+CD38^{-/lo}CD19^+$ cell is the candidate LSC and that the TEL-AML1 fusion acts as a first-hit mutation by endowing the pre-leukemic TEL-$AML1^+$ cells with altered survival and self-renewal properties.

2.3 Candidate LSCs in Chronic Lymphoproliferative Disorders

Chronic lymphoproliferative disorders, including plasma cell dyscrasias, are considered to arise from the transformation of lineage-committed lymphocyte populations, therefore the leukemia-initiating cell in these cases is not considered to have stem cell-like properties. The same is considered to be true in the case of chronic lymphocytic leukemia (CLL), although the cell of origin for CLL has not been formally identified. Indeed, as CLL can be divided almost

exclusively into two prognostic subsets based on the mutational status of immunoglobulin variable heavy chain (V_H) genes (Damle et al. 1999; Hamblin et al. 1999), it was suggested that there may be two distinct cells of origin for CLL: one derived from pre-germinal center (GC) naïve B cells carrying unmutated V_H genes (unmutated CLL), the other derived from post-GC memory B cells carrying mutated V_H genes (mutated CLL) (Stevenson and Caligaris-Cappio 2004). However, gene expression profiles of unmutated and mutated CLL have been shown to be broadly similar both to each other and to antigen-experienced memory B cells (Klein et al. 2001). These studies therefore strongly suggest that the CLL cell of origin is a memory B cell. However, a small study of peripheral blood samples taken from CLL patients revealed the presence of a $CD19^+$ $CD5^+$ population of cells that efflux Hoechst 33342, thus exhibiting the 'side-population' phenotype characteristic of stem cells (Foster et al. 2006). This side-population was not identified in normal healthy donors and identifies a putative stem cell population within CLL. Recent work from our own laboratory has generated a mouse CLL-like model system, by expressing a plasmid-encoding dominant-negative protein kinase Cα (PKCα-KR) in HSCs derived from wild-type mice and then culturing these cells in B-cell generation systems in vitro and in vivo. This resulted in the formation of a population of cells that bear hallmark characteristics of human CLL cells with the phenotype $CD19^{hi}$ $CD5^+$ $CD23^+$ IgM^{lo}, arrest at G_0/G_1 phase of the cell cycle ex vivo, and resistance to apoptosis (Nakagawa et al. 2006; Michie and Nakagawa, unpublished observations). Moreover, PKCα-KR-expressing HSCs possessed an enhanced proliferative capacity both in vivo and in vitro, potentially reflecting the dynamic cellular kinetics that exist during the progression of human CLL (Messmer et al. 2005). Clearly further studies are required to determine whether CLL can be considered a stem cell-mediated disease.

3 Committed Progenitor Cells as Candidate LSCs

The studies described above for AML, CML, and ALL show that, depending on the type of leukemia and its stage or subtype, the candidate LSC may be an HSC or a more committed progenitor which has acquired the potential to self-renew. In addition to identification of the granulocyte-macrophage progenitor as the candidate LSC in blast crisis CML (Jamieson et al. 2004), the acquisition of self-renewal potential by committed progenitor cells has been confirmed in two murine models of AML (Cozzio et al. 2003; Huntly et al. 2004). These important studies showed that transduction with either the *MOZ-TIF2* or *MLL-ENL* oncogene resulted in the development of the capacity for self-renewal in purified populations of committed non-self-renewing myeloid progenitors in vitro and rapid induction of leukemia in murine serial transplantation models. Both *MOZ-TIF2* and *MLL-ENL* were cloned from human leukemias and are capable of producing leukemias in murine models. Interestingly, the

study by Huntly et al. also demonstrated that, in the same model, transduction with *BCR-ABL* did not confer self-renewal properties to the cells (Huntly et al. 2004), indicating that secondary mutations are required in addition to *BCR-ABL* for committed progenitor cells to develop self-renewal characteristics as shown by Jamieson et al. (Jamieson et al. 2004). The mechanisms underlying this difference in the ability of oncogenes to bestow self-renewal properties on a leukemia cell remain to be elucidated.

4 Targeting Self-Renewal in LSC: Potential Therapeutic Strategies

The understanding of self-renewal in normal and leukemic HSC is a rapidly expanding field of research at present. The pathways involved in the self-renewal of HSC and LSC are broadly similar and include the Wnt, Hedgehog, and Notch pathways, the NF-κB, HOX, and polycomb gene families, PTEN and telomerase.

4.1 Wnt Signaling in Leukemia

β-catenin is central to the Wnt signaling pathway. Wnt stimulation results in accumulation of β-catenin and its translocation to the cell nucleus where it interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF1) to regulate genes which are important in embryonic development and cell proliferation. There is increasing experimental evidence that the development of both myeloid and lymphoid leukemias may be dependent on Wnt signaling. As described above (Jamieson et al. 2004), in blast crisis CML compared to earlier stages of disease, the committed granulocyte-macrophage progenitor cell acquires the ability to self-renew in vitro in association with activation of β-catenin, a protein of the WNT signaling pathway which is associated with cell differentiation, proliferation, and death. Furthermore, very recently, research has shown that progression to blast crisis CML is associated with missplicing of GSK-3β in granulocyte-macrophage progenitors, allowing unphosphorylated β-catenin to contribute to self-renewal (Abrahamsson et al. 2007). More recent studies in normal HSC and both CML and AML have demonstrated a central role for Wnt signaling in normal hematopoiesis and leukemogenesis. Zhao et al. demonstrated that although β-catenin-deficient mice can form HSCs, the cells produced are unable to maintain hematopoiesis in the long term (Zhao et al. 2007). In addition, the absence of β-catenin prevented mice developing BCR-ABL^{+} CML, but not BCR-ABL^{+} ALL, suggesting that β-catenin may be preferentially required for leukemias that originate in HSCs but not committed progenitors.

In AML, studies have shown that there is constitutive activation of the Wnt pathway (Simon et al. 2005) and that translocation products, e.g., AML1-ETO,

PML-RARα, and PLZF- RARα activate the Wnt signaling pathway in hematopoietic cells (Muller-Tidow et al. 2004). Recently it has been shown that LEF1 may be important in leukemogenesis. Petropoulos et al. have demonstrated expression of LEF1 in murine HSC and leukemia cells (Petropoulos et al. 2008). Mice transplanted with bone marrow retrovirally transduced to express LEF1 or a constitutively active LEF1 mutant had serious abnormalities of normal hematopoietic differentiation and developed B-ALL and AML, with the LSC exhibiting lymphoid characteristics. This study demonstrates the importance of normal LEF1 expression for routine hematopoietic development. Additional studies suggest that Wnt signaling may also be important in the development of some ALL subtypes, with inhibition of Wnt16 resulting in apoptosis in ALL cells containing the E2A-Pbx1 fusion protein (Mazieres et al. 2005).

Interestingly, two inhibitors of the WNT signaling pathway have recently been described which show efficacy in myeloid leukemias (Kavalerchik et al. 2006; Guzman et al. 2007a). The first of these, MCC-001, a marine sponge-derived β-catenin antagonist, was demonstrated to inhibit the re-plating capacity of CML stem cells, derived from patients with advanced phase CML, at doses which were non-toxic to normal HSCs (Kavalerchik et al. 2006). The second compound, 4-benzyl, 2-methyl 1,2,4-thiadiazolidine 3,5-dione (TDZD-8), a GSK-3β inhibitor, induced rapid cell death in both primary AML and blast crisis CML cells (Guzman et al. 2007a). Further studies in a NOD-SCID mouse xenotransplantation model showed a 93% reduction in engraftment with TDZD-8 for AML samples compared with an 11% reduction for normal cell engraftment. Further studies are currently underway to fully elucidate the mechanisms of action of these two novel compounds and further assess their effect on the LSC compartment.

4.2 The Hedgehog Pathway in Leukemia

The hedgehog family consists of three highly conserved homologous proteins, Desert (DHH), Indian (IHH), and Sonic Hedgehog (SHH). Murine studies have demonstrated that hedgehog proteins have a role in the development of many tissues and organs in the embryo and have been associated with a number of different cancers (Chen et al. 2007). Recent microarray studies by our group (Graham et al. 2007) and others (Radich et al. 2006) provide evidence that the SHH pathway is active in CML stem cells and that the SHH pathway becomes progressively more activated from chronic phase, through accelerated phase to blast crisis (Radich et al. 2006). Furthermore, in this study, activation of the SHH pathway correlated with CD34 expression, suggesting upregulation within CML stem and primitive progenitor cells. In addition, very recent in vitro and in vivo studies have shown that BCR-ABL enhances self-renewal of HSC by activating the SHH pathway via upregulation of smoothened (Dierks et al. 2007). Abnormal Hedgehog signaling may also be a feature of AML and

MDS (Merchant et al. 2007). It is anticipated that future studies will further investigate the role of the Hedgehog pathway in the development of leukemia. Small-molecule inhibitors which target the Hedgehog pathway (e.g., smoothened inhibitors) are in pre-clinical development and may be an effective tool to reduce the malignant stem cell pool.

4.3 The Notch Pathway in Leukemia

Notch family members act as receptors for a signal transduction pathway that controls development and tissue homeostasis by regulating cell fate and differentiation (Artavanis-Tsakonas et al. 1999). Four Notch receptors (NOTCH1–4) and five ligands are known. The ligands initiate Notch signaling by proteolytic cleavage of a metalloprotease called γ-secretase from the intracellular domain of the Notch receptor. Increased expression of constitutively activated NOTCH1 in HSC completely inhibits B-cell development. A gain-of-function mutation is very common in the NOTCH1 receptor in T-ALL. This increased NOTCH signaling results in increased T-cell differentiation and self-renewal in hematopoietic progenitors, leading to transformation to T-ALL (Aster et al. 2008). Notch inhibitors are already in clinical development. MK-0752, a γ-secretase inhibitor, has already entered a Phase 1 clinical trial in patients with T-ALL and other leukemias and the results of this study are awaited (DeAngelo et al. 2006).

4.4 NF-κB in Leukemia

NF-κB is a transcription factor with anti-apoptotic activity which has abnormal expression in both myeloid and lymphoid leukemias (Kordes et al. 2000; Guzman et al. 2001). NF-κB is constitutively activated in LSCs in AML and recently studies have focussed on attempting to eradicate AML stem cells using proteasome inhibitors which induce apoptosis in AML stem cells in association with inhibition of NF-κB and activation of p-53-related genes (Guzman et al. 2002). In addition, the naturally occurring small-molecule inhibitor parthenolide also causes apoptosis in LSC in AML and blast crisis CML, again through inhibition of NF-κB and activation of p-53 and also increased production of reactive oxygen species (Guzman et al. 2005, 2007b).

4.5 The HOX Gene Family in Leukemia

The homeodomain-containing transcription factors of the HOX family are preferentially expressed in primitive self-renewing hematopoietic progenitors and are downregulated after differentiation. HOX genes are important in normal

hematopoiesis, self-renewal, and leukemogenesis (Pineault et al. 2002). Many HOX genes have been linked with the development of acute leukemia, and chromosomal translocations between NUP98 and HOXA9 or HOXD13 are reported in AML (Borrow et al. 1996; Raza-Egilmez et al. 1998), with overexpression of HOXA9 carrying a particularly poor prognosis (Golub et al. 1999). Overexpression of HOX11 has been reported in T-ALL (Hatano et al. 1991).

The Mixed Lineage Leukemia (*MLL*) gene is an upstream regulator of the HOX genes and more than 50 *MLL* gene rearrangements have been described with different transcription partners (Krivtsov and Armstrong 2007). It is believed that the resulting fusion proteins initiate leukemic transformation through the upregulation of HOX genes (e.g., HOXA9 and co-factor MEIS1). It has been proposed that self-renewal in LSCs may be regulated by HOX-dependent pathways (Argiropoulos and Humphries 2007).

4.6 The Polycomb Gene BMI-1 in Leukemia

The polycomb RING-finger protein BMI-1 is an epigenetic chromatin modifier involved in gene repression and is essential for regulating the proliferation of HSCs and LSCs. In a murine model of AML, the LSCs of *Bmi-1*-deficient mice did not result in long-term engraftment and proliferation of cells, but instead, proceeded to terminal differentiation and apoptosis (Lessard and Sauvageau 2003). These effects were completely reversed with the addition of Bmi-1. BMI-1 forms a complex which binds to chromatin and its actions are thought to be mediated via methylation, deacetylation, and ubiquitination of core histones. Therefore, inhibitors of these epigenetic modifications, e.g., the DNA methylation inhibitor 5-azacytidine, histone deacetylase (HDAC) inhibitors, or proteasome inhibitors, could be exploited to inhibit BMI-1 in clinical studies. Proteasome inhibitors, HDAC inhibitors and 5-azacytidine have already been assessed in pre-clinical and clinical studies in AML and MDS (Guzman et al. 2002; Bruserud et al. 2007; Kantarjian et al. 2007; Oki et al. 2007; Plimack et al. 2007; Attar et al. 2008), although there has been little evidence for clinical efficacy in the early phase trials, except for 5-azacytidine in MDS (Kantarjian et al. 2007). HDAC inhibitors induce apoptosis in non-dividing cells and, very recently, Strauss et al. demonstrated that the HDAC inhibitor LAQ824 induced apoptosis in CML progenitor cells when used in combination with the tyrosine kinase inhibitor imatinib (IM) and this was associated with downregulation of MCL-1 (Strauss et al. 2007).

4.7 The Role of PTEN in Leukemia

A recent study has shown that dependence on the tumor suppressor gene PTEN separates HSCs from LSCs (Yilmaz et al. 2006). In an in vivo mouse model,

conditional deletion of PTEN resulted in a myeloproliferative disorder which progressed to acute leukemia over a number of weeks and also induced acute leukemia in recipient mice in a mouse transplantation model. In addition, HSCs were not maintained in the absence of PTEN, as PTEN deletion increased HSC proliferation which resulted in HSC depletion and the cells were unable to reconstitute irradiated mice, i.e., there was loss of self-renewal capacity. It is likely that these effects were modulated by mTOR as rapamycin not only depleted LSCs but also restored normal HSC function, providing a mechanism through which LSCs can be selectively targeted, while maintaining the function of normal HSCs.

4.8 Telomerase in Leukemia

Telomerase is a ribonucleoprotein enzyme which synthesizes telomere repeats and prevents replicative senescence. Studies have shown that telomeres are shorter and telomerase activity higher in CML LSCs compared to normal HSCs (Brummendorf et al. 2000). This raises the possibility of exploiting differences in telomerase activity to target LSCs. A number of clinical studies using agents such as arsenic trioxide, hTERC antisense oligonucleotides, and hTERT vaccines are already underway to modulate telomere dynamics in leukemia (Miller et al. 2002; Vonderheide et al. 2004; Dikmen et al. 2005).

5 The Importance of Developing Targeted Stem Cell Therapies in Leukemia: The CML Story

The importance of developing targeted leukemia therapies is related to the toxicity of conventional chemotherapy regimens to normal cells and the failure of these non-specific agents to target LSCs, resulting in an inability to cure many subtypes of leukemia. The development of molecularly targeted therapies allows specific targeting of cancer cells without affecting normal cells, reducing toxicity, and, in most cases, improving patient quality of life. Although the introduction of IM for the treatment of BCR-ABL-positive malignancies is widely heralded as the first successful molecularly targeted cancer therapy (Druker et al. 2001), it was preceded by others. Perhaps the first real targeted approach to cancer therapy was the use of hormonal manipulation in the form of tamoxifen in breast cancer (Early Breast Cancer Trialists' Collaborative Group 1998). Another was the use of monoclonal antibodies such as the anti-CD20 agent rituximab (Mabthera™) in B-cell disorders (Coiffier et al. 2002). Currently, targeting signal transduction pathways is a major strategy in the development of novel anti-neoplastic agents.

CML represents an excellent model for the study of cancer stem cells because it results from a single genetic mutation (BCR-ABL) and is measurable by

standard laboratory techniques such as FISH and PCR (Kaeda et al. 2002). This also makes CML an ideal disease in which to identify novel agents which target cancer stem cells.

5.1 BCR-ABL Tyrosine Kinase Inhibitors in CML

There have been major advances in the treatment of CML in recent years with the development of IM (Druker et al. 1996; O'Brien et al. 2003) and, more recently, the oral, multi-targeted kinase inhibitor dasatinib (Talpaz et al. 2006) and the second-generation BCR-ABL kinase inhibitor, nilotinib (Kantarjian et al. 2006). IM is a tyrosine kinase inhibitor (TKI) which competitively inhibits ATP binding to BCR-ABL, resulting in inhibition of downstream signal transduction pathways. Despite inducing a complete cytogenetic response in the majority of CML patients in chronic phase (O'Brien et al. 2003; Druker et al. 2006), nearly all patients treated with IM have detectable disease at the molecular level by quantitative RT-PCR (Hughes et al. 2003; Branford et al. 2004) and, therefore, are unlikely to be cured. It has been demonstrated that this molecular persistence results from a population of quiescent CML stem cells which are not effectively targeted by IM (Graham et al. 2002). In addition, a minority of CML patients harbor BCR-ABL kinase domain mutations, rendering them IM-resistant (Gorre et al. 2001; Shah et al. 2002). Dasatinib and nilotinib target IM-resistant mutations (Shah et al. 2004; O'Hare et al. 2005) and, in the case of dasatinib, reach further into the stem cell compartment (Copland et al. 2006). However, despite inhibition of BCR-ABL, quiescent CML stem cells remain insensitive to these compounds (Copland et al. 2006; Jorgensen et al. 2007). Therefore, strategies are required to target both quiescent and proliferating BCR-ABL^{+} cells.

5.2 CML Stem Cell Modeling

Recently, two different dynamic models of CML have been proposed (Michor et al. 2005; Roeder et al. 2006) which arrive at different conclusions. The first model suggests that although IM is a potent inhibitor of differentiated CML cells, it does not reduce the CML stem cell population (Michor et al. 2005). In CML, BCR-ABL transcripts exhibit a biphasic decline in patients responding to IM, but even after years of therapy, the majority of patients have persistent disease at the molecular level (Hughes et al. 2003; Branford et al. 2004). The biphasic decline in BCR-ABL transcripts consists of an initial rapid decline, followed by a slower decrease representing the death of more primitive CML progenitors in response to IM. In support of this first hypothesis, in a proportion of patients that discontinued IM after prolonged treatment and had

achieved a complete molecular response (BCR-ABL negative by RT-PCR), the number of BCR-ABL transcripts rapidly increased over the following 3 months to at least pre-treatment levels (Cortes et al. 2004; Mauro et al. 2004). This indicates that IM does not deplete the CML stem cell population which is maintaining the disease, and supports the hypothesis that CML stem cells are resistant to IM and other TKIs (Graham et al. 2002; Copland et al. 2006; Jorgensen et al. 2007). This model further proposes that, as CML progresses, the number of LSCs rises and the probability of a patient having a resistance mutation also increases as a result of this larger population of CML stem cells (Michor et al. 2005). In addition, it is suggested that the time to treatment failure as a result of acquired resistance is dependent on the growth rate of the CML stem cells. Therefore, based on the theories put forward in this study, IM is extremely unlikely to cure CML patients, and over time, the majority of patients will develop acquired resistance as the stem cell population gradually expands. Thus, the development of strategies to target the LSC population will be vital for the eventual eradication of CML.

The hypothesis proposed in the second study is rather more positive for CML patients (Roeder et al. 2006). It suggests that the clinically observed biphasic pattern of BCR-ABL transcript dynamics may be explained by a selective effect of IM on proliferating CML stem cells. This model makes two main assumptions. First, it assumes that IM inhibits proliferative activity and induces death of proliferating CML stem cells and second, it assumes that there is a large population of quiescent CML stem cells which are resistant to IM due to their quiescent state as previously demonstrated (Graham et al. 2002). However, these quiescent CML stem cells retain the potential for proliferation and are responsible for the rapid relapses seen after stopping IM (Cortes et al. 2004; Mauro et al. 2004). This model predicts that, over time, as quiescent CML stem cells gradually enter the cell cycle, they will proliferate and become sensitive to IM. Therefore, levels of minimal residual disease (MRD) will continue to fall over prolonged periods of IM treatment as suggested by clinical data (Branford et al. 2004), and complete disease eradication may be possible if patients do not develop resistance mutations. The model also proposes that promoting quiescent CML stem cells to enter the cell cycle by using additional agents in combination with IM may enhance the eradication of MRD in CML.

Although these two different hypothetical models of CML dynamics arrive at different conclusions, they both highlight the importance of developing drug combination strategies with IM or the newer agents (dasatinib, nilotinib, and others) to eliminate the quiescent CML stem cell population. To this end, our group has recently shown that the farnesyltransferase inhibitor, BMS-214662, in combination with either IM or dasatinib results in a significant reduction in the CML stem cell population in vitro (Copland et al. 2008) and this combination is now being tested in a murine model, although the exact mechanism of action remains to be elucidated. The use of HDAC inhibitors in combination with IM as described earlier by Strauss et al. also appear to target

the quiescent CML stem cell population (Strauss et al. 2007). Both these combination strategies require further evaluation, but represent realistic potential therapeutic options for the cure of CML in the future.

6 Future Challenges in Targeting LSCs

It is becoming increasingly apparent that the development of agents which specifically target cancer stem cells is vital for the eradication of leukemia and other stem cell-derived malignancies. It is only by understanding the biology of cancer stem cells and developing novel stem cell-directed therapies that progress will be made in eradicating these diseases. At present, a number of pre-clinical strategies are being pursued to target the quiescent LSC. These include the use of self-renewal pathway small-molecule inhibitors, proteasome inhibitors, rapamycin, telomerase inhibitors, 5-azacytidine, HDAC inhibitors, and BMS-214662. In addition, new potential targets and novel small-molecule inhibitors are emerging all the time.

It is likely that a successful strategy for eradicating leukemia will consist of standard chemotherapy agents (e.g., anthracyclines, cytosine arabinoside, fludarabine) to kill the majority of leukemia blast cells in combination with a targeted LSC therapy. The timing of such therapy is likely to be critical and will require careful planning for successful clinical trials. One potential approach would be induction chemotherapy with a combination of standard chemotherapy agents to reduce overall tumor load, followed by LSC-directed therapy when tumor burden is low to eradicate the quiescent LSC population (Fig. 3). For such an approach to be successful, LSC-specific markers must be identified (e.g., CD96 in AML) and sensitive, validated tests (e.g., quantitative RT-PCR for BCR-ABL in CML) must be available to monitor MRD. However, as demonstrated with CML, even the most sensitive molecular tests currently

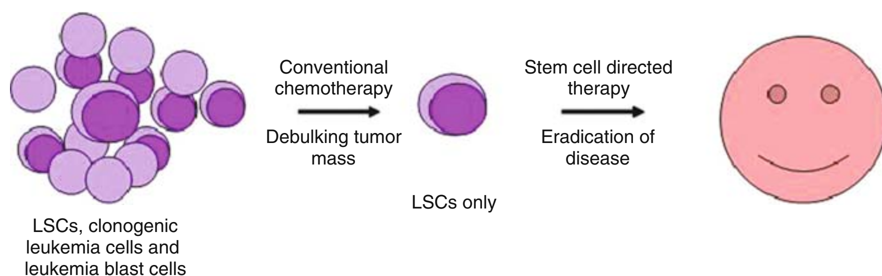

Fig. 3 Schematic diagram of a potential therapeutic strategy to eradicate LSCs. Conventional chemotherapy agents are used for eliminating the bulk of the clonogenic leukemia progenitor cells and non-clonogenic leukemia blast cells. This is followed by a LSC-directed therapy to eradicate the LSC population, which is resistant to conventional chemotherapy, and cure leukemia

available clinically are too insensitive to detect the LSC population, highlighting the importance of understanding LSC biology so that novel stem cell markers and treatments can be identified and more robust measurements of the rare LSC population and its size can be undertaken.

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Prostate Cancer Stem Cells

Elaine M. Hurt, George J. Klarmann, Brian T. Kawasaki, Nima Sharifi, and William L. Farrar

Abstract Prostate cancer is the leading cancer diagnosed in males. Emerging data suggests that cancer arises from only certain cells, termed cancer stem cells, contained within the prostate. In this chapter we will discuss in depth the prostate cancer stem cell. We detail the properties of both normal prostate stem cells and cancer stem cells and discuss the identification and isolation of the prostate cancer stem cells, including the current knowledge of the surface markers used for identification. Furthermore, we discuss the signaling pathways that are important in stem cell maintenance and comment on what is known about these pathways in prostate cancer stem cells and how these pathways, and others, may be targeted to inhibit or kill the tumor-initiating cancer stem cells. In conclusion, we provide a short discussion on the future directions for study of prostate cancer stem cells.

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1 Introduction

1.1 Prostate Cancer and Treatment

Prostate cancer is the leading cancer diagnosis in men in the United States, with 218,890 new cases and 27,050 deaths estimated by the American Cancer Society for 2007 (American Cancer Society 2007). Environmental factors undoubtedly play a key role in the genesis of prostate cancer. Asians, for example, have a higher risk of prostate cancer after they immigrate to North America and adopt a Western diet (Whittemore et al. 1995). Hereditary factors also play an important part. Germline mutations in genes such as RNASEL predispose individuals to a hereditary form of prostate cancer (Casey et al. 2002). Furthermore, multiple genetic polymorphisms with ill-defined functions have a role in the risk of prostate cancer and are additive in risk when they are acquired in combination (Zheng et al. 2008). Therefore, as with most cancers, the events that lead to prostate carcinogenesis are a complex mixture of factors with contributions from hereditary and environmental components (Nelson et al. 2003).

In the development of prostate cancer, androgens, specifically testosterone and the more potent dihydroxytestosterone, are critical and an increase in androgen signaling occurs in the transition from benign prostate to prostate cancer precursors (Tomlins et al. 2007). Prostate cancer is thought to arise from the high-grade pre-neoplastic lesion, prostatic intra-epithelial neoplasia (HPIN). The most convincing evidence for this is that genetic alterations present within prostate cancer are often present within HPIN, supporting this hypothesis (reviewed in Nupponen and Visakorpi 2000; Hughes et al. 2006). Furthermore, recent evidence suggests that most of the expression changes accompanying prostate cancer actually occur during the transition from benign epithelium to HPIN and not HPIN to prostate cancer (Tomlins et al. 2007). HPIN is defined pathologically by the appearance of atypical cells lining the architecturally benign ducts and acini. The diagnosis of prostate cancer relies on the pathological examination of a biopsy of the gland. The microscopic

examination of the biopsy is used to assign a Gleason score to the tumor, which is based upon the architecture of the glands and is predictive of patient prognosis (Gleason 1977). Gleason score ranges from 2 to 10, with a 2 representing small closely packed glands and a good prognosis and 10 representing barely discernable glands with sheets of cells throughout the surrounding tissue and has the worst prognosis (Gleason 1977).

Localized disease is usually treated with surgery (radical prostatectomy) or with radiation therapy or is followed by active surveillance (reviewed in Walsh et al. 2007). A number of minimally invasive options are also under investigation for the treatment of localized prostate cancer, such as high-intensity focused ultrasound, interstitial prostate brachytherapy, and cryotherapy (Barqawi and Crawford 2007). For patients with metastatic disease, the frontline therapy is androgen deprivation therapy (ADT) with chemical or surgical castration (reviewed in Sharifi et al. 2005). The majority of patients in the United States who are treated with ADT undergo chemical castration with gonadotropin-releasing hormone agonists (GnRH-A). Endogenous GnRH is secreted from the hypothalamus which acts on the anterior pituitary resulting in the secretion of luteinizing hormone (LH) into the general circulation. LH acts on the testes to secrete testosterone. The secretion of LH from the anterior pituitary is dependent on the cyclic nature of GnRH action. Constant stimulation of the anterior pituitary with GnRH-A downregulates LH release and hence testosterone release.

Unfortunately, therapy with ADT in the metastatic setting almost always gives rise to castrate-resistant prostate cancer (CRPC). However, this "androgen-independent" prostate cancer is still dependent on the androgen receptor (AR) (reviewed in Sharifi et al. 2006) that has undergone gain-of-function changes resulting in the activation of androgen-responsive genes. These gain-of-function changes in AR are mediated by gene amplification, mutations in the AR, ligand-sensitization of the AR by various growth factors and receptors, as well as by the local conversion of precursors to testosterone and dihydrotestosterone (Scher and Sawyers 2005; Stanbrough et al. 2006).

1.2 The Prostate Gland Architecture and Morphogenesis

The prostate is a small glandular organ that secretes a component of the seminal fluid (Fig. 1). It is composed of three major glandular zones (reviewed in Joshua et al. 2008). The peripheral zone (PZ) surrounds the distal urethra and is the major component of a normal prostate. The central zone (CZ) constitutes about 25% of the prostate and surrounds the ejaculatory ducts. The transition zone (TZ) surrounds the proximal urethra. The supporting stroma is comprised of smooth muscle, fibroblasts, lymphocytes, and neurovascular tissue. Most prostate tumors arise within the PZ with less than 30% arising in the TZ (McNeal et al. 1988). Furthermore, TZ tumors are less aggressive and have lower recurrence rates than PZ tumors.

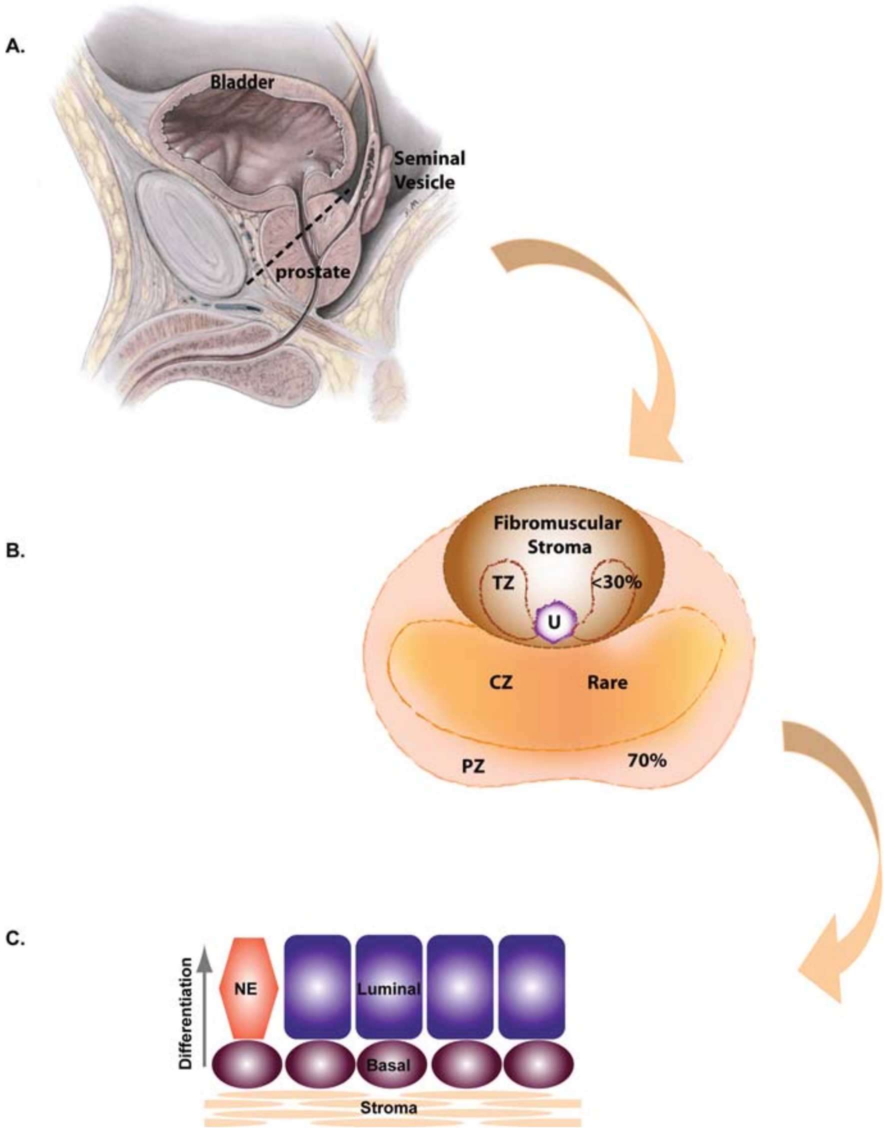

Fig. 1 (**A**) Gross anatomy of the male reproductive tract showing the location of the prostate. LifeART (and/or) MediClip image copyright (2008) Wolters Kluwer Health, Inc./Lippincott Williams & Wilkins. All rights reserved. The *dashed black line* indicates the cross section shown in (**B**) depicting the zones (TZ: transition zone, CZ: central zone, PZ: peripheral zone) and the prevalence of cancer found within each of the zones. U indicates the urethra. A depiction of the cell layers found within the prostate is shown in (**C**). Basal cells give rise to the more differentiated luminal and neuroendocrine (NE) cells as indicated by the *arrow*

The prostate gland forms during gestation as a derivative of the urogenital sinus, in which the epithelial buds protrude and elongate into the surrounding mesenchyme (Risbridger et al. 2005; Thomson and Marker 2006). The buds can bifurcate to form branches with terminal tips, and epithelial cell proliferation primarily occurs at the leading edge. This branching morphogenesis gives rise to the network of secretory ducts, and, in humans, prostate morphogenesis is complete by birth. In rats, the buds are solid epithelial cords that are primarily undifferentiated cells at day 1 that are positive for basal cell markers cytokeratins (K) K5 and K15, and negative for luminal markers K8 and K18 and are low in AR expression (Prins and Birch 1995). As development proceeds basal cells differentiate into luminal cells positive for K8 and AR. The development of the prostate is androgen dependent, with involution occurring upon the withdrawal of androgen and regeneration with restoration of androgen, suggesting that there are androgen-independent prostatic stem cells that survive androgen withdrawal and can regenerate the prostate.

1.3 The Cancer Stem Cell

A malignant tumor is composed of a heterogeneous population of cells with varying degrees of tumorigenic potential, and only a subset of cancer cells can initiate and propagate a tumor. This was first demonstrated in 1961 when researchers' harvested recurrent cancer cells from patients and then autotransplanted the cells. Tumors only formed when injected with up to 1 million cells (Southam and Brunschwig 1961). Other studies showed that only a small fraction of cancer cells could form colonies in vitro (Hamburger and Salmon 1977; Bruce and Van Der Gaag 1963). These observations led investigators to hypothesize that the clonogenic cells arose from cancer stem cells (CSCs) that maintain the rest of the population. However, the stochastic model of tumorigenesis was equally plausible.

The stochastic model states that all cancer cells can proliferate extensively, form colonies in vitro, and initiate new tumors, but only a small fraction has the probability of finding a permissive environment, or niche, for tumor growth (Huntly and Gilliland 2005; Perryman and Sylvester 2006). In contrast, the cancer stem cell model posits that most cancer cells are unable to proliferate extensively, cannot form colonies in vitro, and are unable to initiate new tumors (reviewed in Wicha et al. 2006). Only a rare, biologically unique subpopulation can exhibit these behaviors. The heterogeneous tumor, like normal tissue, is governed by a cellular hierarchy and at the top is the stem cell (Huntly and Gilliland 2005). To prove the latter hypothesis, one would have to isolate these CSCs and show that they exhibit clonogenic capacity whereas their non-cancer stem counterparts do not. Indeed, Lapidot et al. showed that $CD34^{+}CD38^{-}$ cells could be identified as AML stem cells (Lapidot et al. 1994; Bonnet and Dick 1997). NOD/SCID mice injected with $CD34^{+}CD38^{-}$ leukemic cells

developed leukemia whereas those injected with larger numbers of more mature cells ($CD34^+CD38^+$) did not. The leukemia formed in immunocompromised mice resembled the original patient disease in both cellular morphology and their ability to home to the bone marrow. Further studies showed that AML had a hierarchical organization that was similar to normal hematopoietic development (Hope et al. 2004; Bonnet and Dick 1997). Taken together, this was the first series of studies to identify and characterize a CSC. In addition, these experiments also suggested the normal hematopoietic stem cells were targets of transformation into leukemic stem cells. However, later studies began to show some leukemic CSCs in patients were more similar to early progenitors rather than a true hematopoietic stem cell (Tavil et al. 2006).

1.4 Properties of Normal Stem Cells vs. Cancer Stem Cells

A normal stem cell (SC) is defined by its ability to continually repopulate the cells that comprise the organ system. Three properties that enable a stem cell to do this are its differentiation capability (pluripotency), the ability to self-renew, and a high proliferative capacity (reviewed in Tang et al. 2007). The ability of a normal SC to support and propagate an organ or tissue must be tightly regulated. A CSC requires these same properties to sustain and spread a tumor. However, a CSC would not be subject to the same type of genetic regulation as a normal SC (reviewed in Clarke 2005). It is also noteworthy that the term “cancer stem cell” does not necessarily imply its origins are from a SC, as there is the possibility CSCs emerge from early, less-differentiated cells or mature, committed populations.

Pluripotency is the ability of a SC to differentiate into the heterogeneous population of cells that comprise a tissue or, in the case of CSCs, a tumor (reviewed in Lobo et al. 2007). A SC will give rise to a number of different cell types that can be broken down into three groups: fully differentiated cells, transit-amplifying cells, and stem cells (reviewed in Stingl and Caldas 2007). The fully differentiated cells are mitotically inactive cells. They are at the end-stages of cellular differentiation and will never re-enter the active cell cycle phase. The transit-amplifying (TA) cells are fast growing cells that are not fully differentiated. TA cells are able to proliferate for several generations but they eventually terminally differentiate and need to be replenished by the SC (reviewed in Sell 2006). In order to maintain a tissue or tumor a SC must be able to maintain its numbers by giving rise to another, equally pluripotent SC. This property of SC is termed self-renewal.

Self-renewal is the ability of a SC to undergo asymmetrical or symmetrical division (reviewed in Huntly and Gilliland 2005). Asymmetric division forms one daughter and one SC, a mechanism of division is particularly useful to a SC because it generates a TA cell while maintaining its self-renewing capacity. Symmetrical division allows the SC to form two differentiated daughter cells or two SCs. This behavior is critical because it allows the SC to expand its numbers.

When a CSC is transplanted in an immunocompromised mouse, self-renewal and pluripotency are vital for the formation of a tumor that recapitulates the original (reviewed in Wang and Dick 2005). In addition, long-term self-renewal capacity can be measured by serially transplanting the purified CSC population over several generations (reviewed in Huntly and Gilliland 2005). The result is the continual recapitulation of the original tumor phenotype.

Having a high proliferative capacity helps a SC maintain a tissue or tumor. It is worth noting that self-renewal and proliferation are not the same. Self-renewal is a unique cell division that enables the SC to maintain its high proliferative and differentiation capacity as the parental SC (reviewed in Al-Hajj and Clarke 2004). This quality is of particular relevance during hematopoietic development. Work done by Morrison and Weissman (1994) showed that multipotent progenitor cells recapitulated mature blood cells in lethally irradiated mice but they were unable to maintain the system for more than 2 months. The injected cells became more differentiated and lost their ability to proliferate. In contrast, hematopoietic SCs were able to recapitulate the blood system for the life of the animal. In addition, these SCs were serially transplanted into lethally irradiated mice and continued to maintain the hematopoietic system. The SCs maintained their high proliferative capacity and ability to self-renew. CSCs and normal SCs share the ability to self-renew and maintain the capacity to proliferate extensively. They are the putative population responsible for generation and maintenance of a heterogeneous population of cells. Breast cancer was the first example of a solid tumor found to have CSCs. As little as 100 breast CSCs formed tumors in a murine xenograft model (Al-Hajj et al. 2003). Furthermore, these cancer SCs were able to recapitulate the tumor when serially transplanted into NOD/SCID mice up to four passages. The vast majority of cells isolated from the tumors appeared to be transit-amplifying and/or terminally differentiated cells and these cells were unable to generate a tumor. Presumably with every cell division, the transit-amplifying cell became more differentiated and eventually lost its proliferative capacity.

In summary, the existence of CSCs and their ability to differentiate into multiple lineages, self-renew, and high proliferative capacity makes them particularly insidious to the nature of oncogenesis, malignancy, tumor recurrence, and therapies that do not target these cells. These tumor-initiating cells are the tumorigenic force behind tumor initiation, growth, metastasis, drug resistance, and relapse (reviewed in Pardal et al. 2003).

2 The Cell of Origin of Normal and Cancerous Prostatic Stem Cells

The cellular origin of the prostatic SC is still under debate. There are several lines of evidence that the normal prostate SC has a basal phenotype (reviewed in Collins and Maitland 2006). The prostate epithelium is composed of two morphologically distinct layers, the basal and luminal. These layers are further composed of three types of cells: basal, secretory luminal, and neuroendocrine. The luminal cells are terminally differentiated cells that secrete prostate-specific

antigen (PSA) and prostatic acid phosphatase (PAP) and are the majority of cells found within both normal and hyperplastic epithelium. They express low-molecular weight cytokeratins, such as K8 and K18, and (Sar et al. 1990) and are androgen-dependent (Kyprianou and Isaacs 1988). In contrast, the basal cells are less differentiated, do not secrete PSA or PAP, express p63 and high-molecular weight cytokeratins (K5 and K14), have low to undetectable levels of AR, and are androgen-independent (Kyprianou and Isaacs 1988). There are some cells that express a phenotype intermediate between basal and luminal in that they express K5, K8, and K18 but not K14 (Verhagen et al. 1992).

The identification of SCs in the prostate was first demonstrated by English et al.(1987) in rats using androgen cycling experiments where the prostate involutes upon androgen withdrawal and regenerates with return of androgen. They determined that cells within the basal layer contained the cells for regenerating the prostate, i.e., the stem cells. Isaacs and Coffey (1989) proposed the existence of long-lived SCs that are androgen-independent and give rise to the androgen-responsive transit-amplifying cells that in turn produce the androgen-dependent secretory luminal cells. It was further demonstrated that basal cells that rapidly adhere to type I collagen are both clonogenic (Xin et al. 2005; Collins et al. 2001) and capable of forming prostate-like glands in immunocompromised mice (Collins et al. 2001). Several in vitro studies revealed that prostatic basal epithelial cells can give rise to luminal cells (Liu et al. 1997; Robinson et al. 1998; Tran et al. 2002). Mice that are null for *p63*, a basal cell marker, are born without a prostate, providing compelling evidence that the SC is part of the basal component (Signoretti et al. 2000, 2005). In the case of prostatic CSCs, several groups have reported that the cells displaying SC characteristics are AR negative and express basal cytokeratins (Collins et al. 2005; Richardson et al. 2004; Patrawala et al. 2006).

However, there also exists evidence that the prostatic SC may have a TA or even a luminal cell origin. Since the majority of cells present in prostate cancers are of the luminal type and there are often very few detectable basal cells in adenocarcinomas, it was suggested that prostate cancers most often arise from a luminal cell (Nagle et al. 1987). It was suggested that intermediate TA cells that have regained the ability to self-renew are responsible for the generation of prostate cancer (van Leenders and Schalken 2001). Others showed the prostatic SCs may not be limited to the basal compartment. Long-term BrdU label retention experiments label cells of both basal and luminal origin even after several cycles of androgen ablation, suggesting that the SC is not restricted to the basal layer (Tsujimura et al. 2002).

3 Identification and Isolation of Cancer Stem Cells

The first step in understanding these cells is to definitively identify them. The isolation of prostate CSCs has taken three major approaches. Two of the approaches, side populations (SP) and sphere formation, are based on biological

aspects of the cells while the third approach is to identify the CSCs by surface markers. All of these approaches rely heavily on principles first determined in both embryonic and hematopoietic SCs. An overview of the characteristics of CSCs, TAs, and differentiated cells discussed below is provided in Fig. 2.

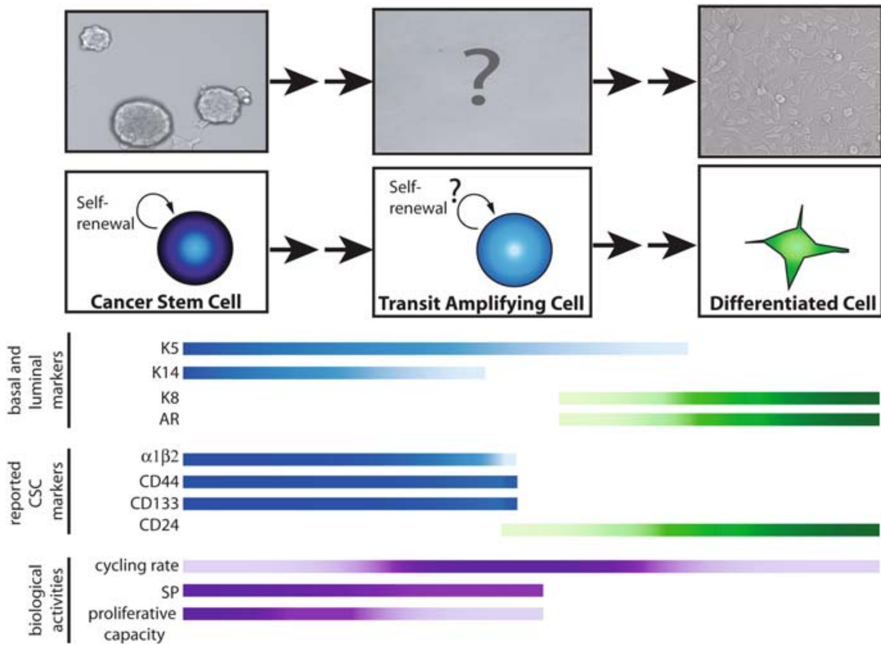

Fig. 2 A model depicting the hierarchical organization of prostate cancer stem cells, transit-amplifying cells, and differentiated cells. Cell markers and biological properties used to identify various populations are shown, where a *darker color* indicates a higher expression/function and a *lighter color* represents the loss of expression/function. The pictures shown across the *top* show the morphology of the cells grown in culture

3.1 Side Populations as a Means of Stem Cell Enrichment

The first approach has been to measure efflux of the Hoechst 33342 fluorescent dye, a substrate for ATP-binding cassette (ABC) transporters, out of the cells. Based on the observations that hematopoietic stem cells overexpress the ABC transporter, ABCG2 (Kim et al. 2002), and that expression of this drug transporter inhibits differentiation of stem cells (Zhou et al. 2001), it was hypothesized that the CSC should have higher expression of drug transporters, namely those responsible for multi-drug resistance. There is convincing evidence linking the ABCG2 phenotype to the SP population, since bone marrow cells of *abcg2–/–* mice lack a SP (Zhou et al. 2002). The isolation of cells based on their

ability to efflux Hoechst 33342 was first observed using bone marrow aspirates by Goodell et al. (1996). While simultaneously displaying the Hoechst fluorescence at two emission wavelengths (675 and 450 nm) they identified a small subpopulation of whole bone marrow cells that were unstained; they termed the SP. They further determined that these cells contained SC characteristics and had a 1000-fold increase in the ability to repopulate the bone marrow.

The SP was shown to exist in prostate epithelial cells isolated from men undergoing surgery for bladder outflow obstruction as a result of benign prostate disease (Bhatt et al. 2003). The SP accounted for approximately 1% of prostate epithelial cells. Furthermore Bhatt et al. showed that this SP was significantly reduced in the presence of verapamil, an inhibitor of ABC transporters, and that the SP was comprised of both cells in G_0 and those entering G_1 of the cell cycle. It was the conclusion of Bhatt et al. that the SP contains both the prostatic SCs and the TA cells. The SCs were identified by (1) the efflux of Hoechst 33342, (2) their quiescent nature (i.e., cells in G_0), and (3) the expression of the basal marker integrin-α2, while the transit-amplifying cells could be identified by (1) the efflux of Hoechst 33342, (2) cycling cells (i.e., cells entering G_1), and (3) expression of both the basal marker integrin-α2 and the luminal marker K8.

In prostate cancer cell lines, LAPC-9 xenograft tumors contained a detectable SP but there was no detectable SP in DU145, LNCaP, PC3, and PPC-1 cell lines (Patrawala et al. 2005). It was also determined that the SP cells of LAPC-9 were more tumorigenic than the non-SP cells, with as few as 100 SP cells giving rise to tumors (25% incidence), whereas 300,000 non-SP cells were needed to generate a tumor. Since the ABCG2 transporter was implicated as responsible for the SP phenotype, Patrawala et al. isolated $ABCG2^+$ and $ABCG2^-$cells from the DU145 prostate cancer cell line and showed that they were similarly tumorigenic. Furthermore $ABCG2^+$cells did not show increased expression of genes known to play a role in SC maintenance (i.e., *Notch, smoothened, Oct-3/4,*and *β-catenin*). Thus, they concluded that the SP is enriched for CSCs, but that it contains not only the most primitive of stem cells but also the more differentiated TA cells.

Currently, most investigators agree that the SP represents an enrichment of CSCs but that it is not a pure population (reviewed in Hadnagy et al. 2006). It is widely believed that this population contains not only the more primitive SC but also TA cells or cells further along the differentiation pathway.

3.2 Enrichment of Prostatic CSCs by Culturing Non-adherent Spheres

A second biological approach to isolate prostatic CSCs is based on their ability to form spheres when plated in clonal numbers. This takes advantage of several principles that were identified in other normal and cancer stem cells. For

example, in neural cells it was found that a population of undifferentiated cells was maintained by culturing the cells in a non-adherent condition. These neurospheres are enriched for stem and progenitor cells and contain cells that are multipotent (Reynolds and Weiss 1996). Using the results obtained with neural cells, Dontu et al. established that breast CSCs can be enriched by plating cells in serum-replacement media on ultralow attachment plates (Dontu et al. 2003a). The enrichment of breast CSCs by culture conditions favoring SC survival and self-renewal has been used to further characterize these cells (Dontu et al. 2003b, 2004; Liu et al. 2006). In prostate cancer, it was demonstrated that purified prostate CSCs also grow as prostataspheres when maintained in culture (Patrawala et al. 2006; Hurt et al. 2008), however, until recently this approach was not used as a means to enrich prostate cancer stem cells for further study.

Recently, this approach was utilized by Li et al. where they were showed that holoclones generated from the PC3 prostate cancer cell line were enriched for tumor-initiating cells that could be serially transplanted in NOD/SCID mice (Li et al. 2008). The term holoclone has been used in the keratinocyte literature, where single cell suspensions of keratinocytes give rise to three types of colonies: the holoclone which is highly enriched for cells that do not differentiate (abort), the paraclone which can only be maintained for a short number of passages before it differentiates, and the meroclone which is a mixture of the other two types of clones (Barrandon and Green 1987). Morphologically the PC3-derived holoclones are similar to the prostataspheres that were grown from purified CSCs. The PC3-derived holoclones also expressed high levels of CD44 and integrin-$\alpha2\beta1$, markers that also identify prostatic CSCs. Since this research was done with a single cell line, further studies need to be conducted to determine if enrichment of prostatic CSCs can be achieved through this culture system.

3.3 Identification of Prostatic Cancer Stem Cells Through Surface Markers

One method for the identification of CSCs utilizes cell surface markers as a means for identification and isolation. Most surface markers used to date have been selected based on an understanding of where the SCs may be located (basal cell markers) or from an understanding of the important markers in both hematopoetic and embryonic SCs.

3.3.1 Normal Prostate Stem Cell Markers

Collins et al. (2001), taking advantage of the known association of SCs with basement membranes, identified integrin-$\alpha2\beta1$ cells in normal prostate that showed increased colony-forming ability when compared to the total basal cell population. Further characterization of these cells determined that they

were also CD133$^+$ (Richardson et al. 2004). Furthermore, they showed that the CD133-positive cells are restricted to the $\alpha 2\beta 1^{hi}$ cells. The $\alpha 2\beta 1^{hi}$ CD133$^+$cells were capable of regenerating prostate like acini in immunocompromised mice and demonstrated a high proliferative potential in vitro (Richardson et al. 2004).

Another basal cell marker, CD44, was also reported to mark prostate stem cells (Liu et al. 1997). Liu et al. demonstrated that CD44$^+$ prostate epithelial cells when co-cultured with stromal cells in the presence of Matrigel and androgen were capable of producing PSA, a luminal marker. This presumably occurred through differentiation, although it was not formally proven.

3.3.2 Prostate Cancer Stem Cell Markers

Using cell lines, Patrawala et al. demonstrated that purified CD44$^+$cells exhibited important properties of CSCs, such as (1) long-term retention of BrdU indicating that the cells are relatively quiescent, (2) initiation of tumors at low numbers (100 cells), (3) the tumors could be serially transplanted, and (4) were maintained in cell culture for extended periods of time (Patrawala et al. 2006; Tang et al. 2007). However, it was these authors who concluded that the CD44$^+$ population was still a heterogeneous population that was enriched for CSCs.

Working with patient primary samples, Collins et al. demonstrated CD44$^+$CD133$^+$integrin-$\alpha 2\beta 1^{hi}$cells had higher proliferation rates and invasive potentials compared with CD133-negative cells. This population represented approximately 0.1% of tumor cells, independent of Gleason grade. Furthermore, these cells could differentiate into AR-positive cells and cells that recapitulated the original tumor phenotype in in vitro differentiation cultures. The tumorigenic capacities of these cells were not tested, which is often considered a vital element in proving that the cells under investigation are indeed CSCs. However, using the DU145 cell line, Chen et al. demonstrated that the CD44$^+$CD133$^+$integrin-$\alpha 2\beta 1^{hi}$cells are tumorigenic (Wei et al. 2007).

Also using cell lines, Hurt et al. (2008) demonstrated that rare CD44$^+$CD24$^-$ cells (0.4% of the total population) are the tumorigenic cells. Remarkably, when these cells were depleted from the total cell line, the remaining cells did not initiate tumor formation. Furthermore, CD44$^+$CD24$^-$ cells could be maintained in culture and tumors removed from immunocompromised mice injected with 1000 CD44$^+$CD24$^-$ cells were phenotypically similar to tumors removed from mice injected with 1×10^6 of the total cell line. Importantly, it was demonstrated that these cells contained a molecular signature (termed the invasiveness gene signature, IGS) derived from breast CSCs and predicted poor survival in not only breast cancer but prostate cancer patients (Liu et al. 2007). Therefore, patients with a poor prognosis may indeed have CSC-enriched tumors leading to an aggressive clinical course. Hurt et al. also demonstrated that CD44$^+$CD24$^-$ contained higher levels of CD133 expression in comparison to the non-CSC population, such that this population was in fact CD44$^+$CD24$^-$CD133$^+$.

Therefore, the most primitive prostatic CSCs appear to be CD44^{+}CD133^{+} integrin-$\alpha2\beta1^{hi}$CD24^{-}. However, more extensive experiments need to be conducted using primary samples to confirm that this is an accurate phenotype in prostate cancer patients.

4 Signaling Pathways Important in Prostate CSCs

By definition, both normal and CSCs must maintain self-renewal capacity in addition to giving rise to differentiated progeny cells, and thus there is likely to be similarity in the pathways governing these processes between normal and CSCs. Understanding the signaling pathways and the molecular mechanisms that are responsible for regulating these events in CSCs is extremely important, as it is likely that tumor growth is determined in part by disregulation of the Wnt, hedgehog, Bmi-1, and/or Notch signaling pathways (Lobo et al. 2007). An overview of the pathways discussed below can be found in Fig. 3.

4.1 Wnt/β-Catenin Pathway

The Wnt/β-catenin pathway is important for cell proliferation, differentiation, and self-renewal in hematopoietic stem cells (reviewed in Mohinta et al. 2007). In addition, defects in Wnt signaling are associated with several tumor types including colon, skin, breast, prostate, and bone marrow (Taipale and Beachy 2001; Mohinta et al. 2007; Bastian et al. 2005; Bruxvoort et al. 2007; Reya et al. 2003). Wnts are a 19-member family of secreted glycoproteins that bind to several different cell surface receptors and determine signal transduction by the canonical or non-canonical pathway (Cadigan and Nusse 1997). Wnt binding to LRP5/6 and Frizzled receptors on a Dishevelled (Dvl) platform inhibits GSK-3β, which, in the absence of Wnt binding, is found in complex with axin and APC and phosphorylates β-catenin, marking it for proteolytic degradation. Accumulation of free β-catenin in the cytoplasm and nucleus is followed by direct association with the transcription factors TCF/LEF, resulting in subsequent activation of Wnt target genes that involve epithelial-to-mesenchymal (EMT) transition and cell proliferation (e.g., c-myc, cyclin D1, and CD44). In addition, β-catenin is a coactivator of AR-mediated transcription activity, which suggests a crosstalk between Wnt and androgen signaling in prostate cancer (Yang et al. 2002; Terry et al. 2006). Several secreted antagonists help regulate Wnt signaling to prevent overactivation. WIF1 and SFRPs block Wnt/Frizzled binding, and DKK proteins interfere with LRP5/6 (Kawano and Kypta 2003). In many cancers including prostate, these Wnt inhibitors are downregulated through both genetic and epigenetic changes (Wissmann et al. 2003; Kawano et al. 2006), resulting in upregulation of the Wnt pathway.

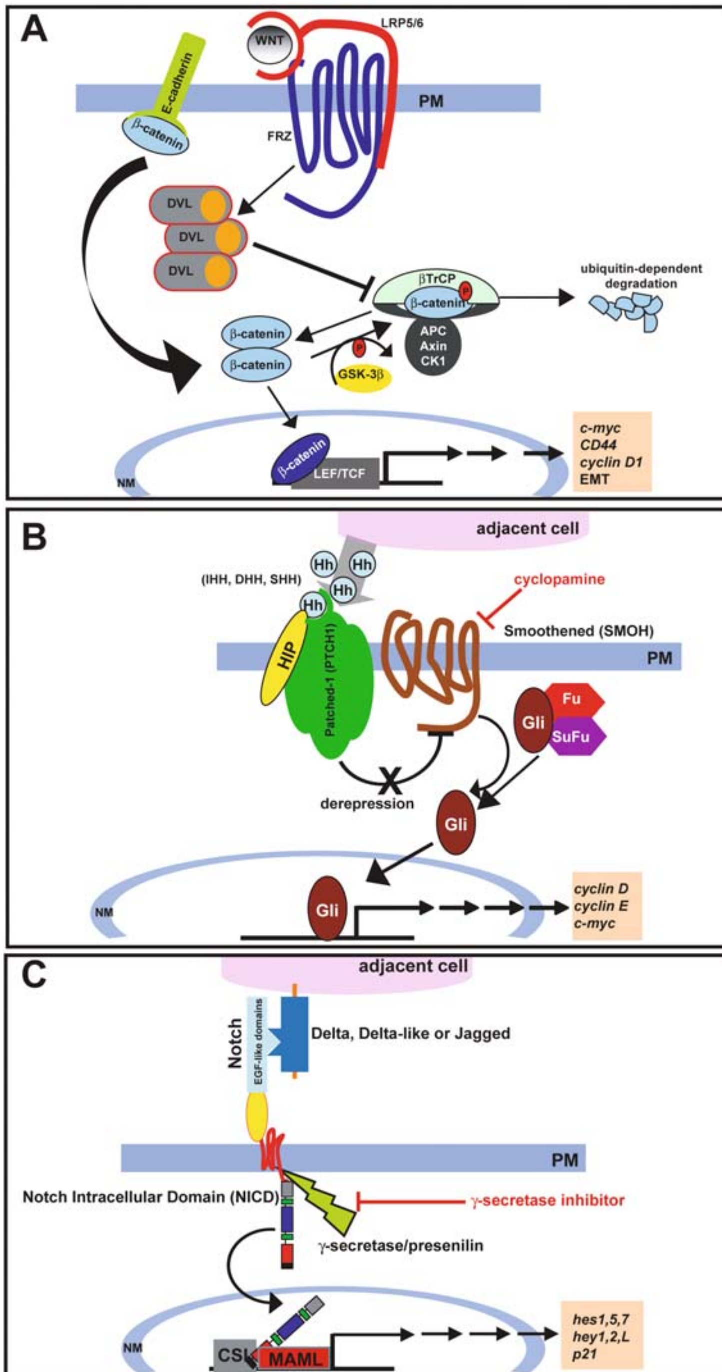

Fig. 3 A schematic of the (**A**) Wnt/β-catenin pathway, (**B**) sonic hedgehog pathway, and (**C**) Notch pathway. Details are provided in the text

The Wnt pathway is also associated with development of bone metastases associated with prostate cancer (reviewed by Hall and Keller 2006). Data indicate that the Wnt antagonist DKK-1 is a switch that controls bone metastases from osteolytic to osteoblastic in a manner depending on the tumor microenvironment. While it is largely unknown what role, if any, that Wnt signaling plays in normal prostate development, recent data support its role in prostate CSCs. Two independent studies demonstrated increased *β-catenin* gene expression in cell line-derived $CD44^+$ CSCs relative to the non-stem cell population (Patrawala et al. 2006; Hurt et al. 2008). In addition, the Wnt pathway antagonist *DKK1* is downregulated in the $CD44^+$ CSCs (EMH unpublished observations). Though these data suggest that Wnt signaling is increased, it was not determined whether β-catenin was localized in the nucleus, where it is required for TCF/LEF activation. Additional studies are needed to determine how upregulated Wnt signaling contributes to the CSC phenotype.

4.2 Hedgehog Signaling

The hedgehog gene family encodes several secreted glycoproteins such as Indian hedgehog (IHh), desert hedgehog (DHh), and sonic hedgehog (SHh). These serve to mediate signaling in embryogenesis and development through activation of the GLI family of transcription factors (reviewed in Taipale and Beachy 2001; Liu et al. 2005) The Hh pathway is somewhat unique in that the signals serve to relieve a series of repressive interactions. The receptor for Hh, the transmembrane protein patched 1 (PTCH), normally binds and inhibits smoothened (SMOH), a G-protein-coupled receptor that is related to FRZ. When secreted Hh binds both PTCH and hedgehog-interacting protein (HIP), SMOH initiates a transcriptional response. Specifically, SMOH activates the serine/threonine kinase Fused (Fu) to release GLI from the sequestration by Suppressor of Fused (SuFu). Subsequently GLI proteins are able to translocate to the nucleus and regulate transcription of *cyclin D and E, c-myc*, and other genes involved in cell proliferation and differentiation (reviewed in Nybakken and Perrimon 2002; Pasca di Magliano and Hebrok 2003).

Normal mammalian prostate development requires functional Hh signaling (Berman et al. 2004; Freestone et al. 2003). In rodent models, SHh is expressed during formation of prostate ducts and branches from epithelial buds (Lamm et al. 2002; Berman et al. 2004). Recently, Hh expression was confirmed in human fetal prostate development (Zhu et al. 2007). In mature prostate cells, Hh expression is low (Zhu et al. 2007), which suggests that it is only during development and differentiation, steps that involve stem or TA cells, that Hh is required. In addition, *SHh* and *IHh* expression increases in prostate cancer cells (Sanchez et al. 2004; reviewed in Anton Aparicio et al. 2007), and the Hh targets of *PTCH* and *GLI*are upregulated in human prostate cancer cells but not in normal samples (Karhadkar et al. 2004). Thus, activation of the Hh pathway is an important component in the unregulated growth of prostate cancer cells.

Several lines of evidence suggest that Hh signaling is also important in prostate CSCs. One study found that *SMO* mRNA is more highly expressed in xenograft tumors initiated from CD44$^+$ stem cell-like subpopulation in DU145, LAPC4, and LAPC9 human prostate cancer cell lines relative to CD44$^-$ cells (Patrawala et al. 2006). A more recent study using microarrays found that *SHH*and*SMOH* genes are expressed at higher levels in CD44$^+$CD24$^-$ LNCaP tumor-initiating cells compared with the non-tumorigenic CD44$^+$CD24$^-$ depleted population, while the SMOH inhibitor *PTCH* is downregulated (Hurt et al. 2008). In addition, enforced overexpression of GLI in normal primary prostate progenitor-like cells confers unlimited growth, while daily injection of the Hh pathway inhibitor cyclopamine completely represses xenograft tumors (Karhadkar et al. 2004). Even 5 months after halting cyclopamine treatment, tumors failed to regrow, suggesting that tumor-initiating cells require Hh signaling and that these cells were killed by cyclopamine. These reports support the notion that the Hh pathway is a key component in CSC maintenance and also suggest the exciting possibility some prostate CSCs may originate from a normal prostate SC that acquires an upregulated Hh pathway (Karhadkar et al. 2004).

The polycomb group transcription factor protein Bmi-1 is a key regulator in self-renewal in hematopoietic, leukemic, and neural SCs (reviewed in Grinstein and Wernet 2007; Liu et al. 2005). Bmi-1 is a transcriptional repressor of p16 and INK-4A/ADP ribosylation factor (ARF), important cell cycle regulatory genes linked to cancer (Molofsky et al. 2003). Recently it was shown that the Hh pathway in human breast CSCs upregulates Bmi-1 and that Bmi-1 promotes mammosphere growth and thus CSC self-renewal and proliferation (Liu et al. 2006). This report suggests that the effects of Hh signals in stem cells may in fact be mediated by Bmi-1 (Liu et al. 2006). Consistent with its role in other CSCs, Bmi-1 is also preferentially expressed in LNCaP and other prostate tumor-initiating cells (Hurt et al. 2008; Patrawala et al. 2006) compared with the non-stem cell population.

4.3 Notch Signaling

Notch signaling is important in regulating cell fate determination in developing tissue, for cell proliferation and for maintaining SC self-renewal (reviewed in Weng and Aster 2004; Liu et al. 2005) (Fig. 2C). Mammals have four Notch proteins, Notch 1–4, that are transmembrane receptors existing as a heterodimer pro-form. These interact with surface ligands such as Delta, Delta-like, and Jagged (DSL ligands) from another cell. This interaction triggers a series of proteolytic events by ADAM (a disintegrin and metalloproteinase) and γ-secretase/presenilin that ultimately release the intracellular Notch domain, which translocates to the nucleus and binds to the transcription factor CSL and transcriptional activators of the mastermind-like family. This ternary complex binds to genes containing CSL-binding sites and activates their transcription.

The Notch pathway is linked to both oncogenic effects and tumor suppressor functions (Weng and Aster 2004). Jagged-1 is overexpressed in metastatic prostate cancer (Santagata et al. 2004), and knockdown experiments revealed that its loss inhibits prostate cancer cell growth and forces S phase cell cycle arrest (Zhang et al. 2006). In addition, the levels of *Notch1* expression are greater in murine prostate tumor cells, but constitutive expression of the active form of Notch1 inhibits DU145, LNCaP, and PC3 cell line proliferation (Shou et al. 2001). Thus, the effects of Notch signaling may depend on the timing and level of activation within the cell.

Studies have shown that Notch signal transduction is important to normal prostate epithelial cell proliferation and differentiation (Wang et al. 2004, 2006; Shou et al. 2001). *Notch1* is expressed in basal epithelial prostate cells (Shou et al. 2001), and transgenic mouse models in which Notch-expressing cells can be specifically ablated reveal that prostate branching morphogenesis, growth, and differentiation of early post-natal prostate cells in culture are all inhibited. Furthermore, prostate re-growth in castrated mice requires Notch1 expression (Wang et al. 2004). A recent study used γ-secretase inhibitors to block Notch maturation of neonatal prostate cells in culture and evaluated *Notch1* conditional knockout mice (Wang et al. 2006). Their data indicate that loss of Notch signaling increased epithelial progenitor cell proliferation and impaired differentiation, suggesting that prostate progenitor cell proliferation is negatively regulated by Notch. These progenitor cells also express K8 and K14, markers of normal prostate stem and TA cells (Rizzo et al. 2005). Thus, proper control of the Notch pathway appears to be important to regulate a balance between SC maintenance and activation of differentiation within the prostate. It is likely that Notch serves the same function in prostate CSCs, as microarray data from $CD44^{+}CD24^{-}$LNCaP tumor-initiating cells confirm a reduced expression of *Notch 1–3* and *Jagged 1* relative to the non-stem cell population (EMH unpublished observations). Interestingly, Notch1 was preferentially expressed in prostate cancer side population cells (Patrawala et al. 2005).

4.4 Nanog, Oct3/4, and Sox2 Transcriptional Network

The transcription factors Nanog, Oct3/4, and Sox2 are important for self-renewal and inhibition of differentiation in embryonic stem (ES) cells (Pan and Thomson 2007). The expression of Oct3/4 and Nanog can be sustained by, among other mechanisms, the Wnt pathway (Sato et al. 2004). Furthermore, the transcription factors Tcf3, which is downstream in the Wnt pathway, and p53 limit Nanog expression to allow the cell to initiate differentiation, and loss of Tcf3 enhances the level of Nanog in ES cells (Pereira et al. 2006). Nanog, a homeobox transcription factor, blocks ES cell differentiation and thus regulates self-renewal. The POU-domain protein Oct3/4 (octamer-binding transcription factor 3/4) can either activate or repress transcription depending on

the promoter sequence context (Pesce and Scholer 2001) and is a key regulator of pluripotency in mouse and human ES cells. These important ES cell transcription factors form an interdependent regulatory network where they can enhance or restrict expression of each other in order to maintain pluripotency and self-renewal (Boyer et al. 2005). This complex regulatory network is likely necessary to ensure proper embryonic development, and which differentiation pathway is activated, and at what stage, may depend on as yet uncharacterized Nanog, Oct3/4, and Sox2 interactions with co-activators or repressors (Babaie et al. 2007). A recent study of telomerase-immortalized primary human prostate cancer progenitor-like cells found *Oct4*, *Nanog*, and *Sox2* genes were expressed (Gu et al. 2007). Moreover, these cells were also AR negative and CD44 positive and were tumorigenic in mice. In addition both Patrawala et al.(2006) and Hurt et al. (2008) reported that $CD44^{+}$ prostate cancer cells are enriched for *Oct3/4* gene expression while *Tcf3* expression was decreased (EMH unpublished observations). These studies strongly suggest that Nanog/Oct3/4/Sox2 network is important in maintaining prostate CSCs. However, the mechanisms controlling these transcription factors and the downstream effects have yet to be fully elucidated in both normal and CSCs.

5 Targeting of CSCs

With the understanding of the molecular events governing CSCs it will be possible to develop therapeutics aimed at them. This is of paramount importance since the CSCs may mediate resistance and relapse of the most aggressive tumors to current treatments. This resistance may in part be the reactivation of several signaling cascades, such as sonic hedgehog, Wnt, Notch, epidermal growth factor (EGF/EGFR) in the CSCs combined with increases in DNA repair mechanisms and ABC transporter-mediated multi-drug resistance (Mimeault et al. 2007a,b; Dean et al. 2005; Galmozzi et al. 2006; Barker and Clevers 2006; Rubin and De Sauvage 2006; Fodde and Brabletz 2007; de Jonge-Peeters et al. 2007; Bao et al. 2006).

5.1 Targeting ABC Transporters

Chemotherapeutic agents are effluxed using mechanisms similar to those for Hoechst 33342 dye. The SP are enriched in prostatic CSCs. Therefore, blocking drug transporters in combination with administration of a chemotherapeutic agent may be effective at inducing cell death of CSCs simply by keeping the chemotherapeutic agent within the cell. The development of ABC transporter inhibitors has been plagued by many problems, including toxicity and adverse pharmacokinetics (Shukla et al. 2008). It was shown that ABCG2 does not identify the tumor-initiating cells in the prostate cancer cell line DU145 (Patrawala et al. 2005),

therefore a better understanding of exactly which transporters are highly expressed in prostate CSCs will also aid in the selection of therapeutic agents.

5.2 Targeting the Sonic Hedgehog Pathway

Cyclopamine targets Smoothened, a G-protein receptor that is kept inactive by Patched. Treatment of mice with xenograft PC3 tumors with cyclopamine resulted in apoptosis and tumor regression and inhibited recurrence of the tumor for 5 months after removal of treatment (Karhadkar et al. 2004). Furthermore, it was shown that treatment of PC3 $CD44^{+}$ CSCs with cyclopamine results in a decreased expression of MDR1 and ABCG2 suggesting that hedgehog signaling may also lead to a decrease in multi-drug resistance (Sims-Mourtada et al. 2007). Therefore, targeting hedgehog for the treatment of prostate cancer may indeed kill the CSC population and result in a greater toxicity to the cells in general by reducing multi-drug resistance.

5.3 Targeting the Notch Pathway

Gamma-secretase inhibitors prevent the formation of an active Notch by inhibiting its cleavage. In the case of AML, the use of a γ-secretase inhibitor (DAPT) inhibited the growth of the AML stem cell ($CD34^{+}CD38^{-}$) (Gal et al. 2006). The search for other γ-secretase inhibitors continue, especially for the treatment for Alzheimer's where this protein is also important in the etiology of the disease. A new inhibitor, LY15039, is currently being investigated for safety, tolerance, and efficacy in the Alzheimer's setting (Siemers et al. 2007). It remains to be determined if the use of γ-secretase inhibitors will have an effect on prostatic CSC, however, the intricacies of Notch signaling, such as an increase in TA cells with the inhibition of Notch (discussed in more detail above), may make this a less desirable target in prostate cancer.

5.4 Targeting the Niche of Stem Cells

Several lines of evidence show the importance of the niche in tumorigenesis. There is often critical interaction between the tumor cells and cells found within the niche, and this has also been demonstrated for CSCs. In brain tumors, the CSCs are located in close proximity of vessels that provide an environment enriched in factors that shelter them from apoptosis and maintain the balance between self-renewal and differentiation (Shen et al. 2004; Ramirez-Castillejo et al. 2006). In this system, it is thought that the endothelial cells that line the blood vessels promote survival and self-renewal. Indeed, Calabrese et al. (2007) demonstrate co-culture of CSCs and endothelial cells results in proliferation of

the CSCs that closely associate with the endothelial cells. In vivo, medulloblastoma cells transplanted with endothelial cells in immunocompromised mice resulted in faster growing and larger tumors than in mice that were transplanted with medulloblastoma cells without endothelial cells. Furthermore, they demonstrated treatment of tumor-bearing mice with anti-VEGF, which abolishes angiogenesis thereby reducing endothelial cells in vivo,decreased the number of CSCs and the growth rate of the tumor. In the case of normal prostatic epithelial SCs, it was shown that they reside proximal to the urethra at a band of smooth muscle cells that secrete TGFβ (Tsujimura et al. 2002). The secretion of TGFβ in this area may be important in maintaining the niche for prostatic CSCs, since this factor is important in promoting quiescence of SCs (Salm et al. 2005). It would be interesting to determine the effect of inhibitors of TGFβ signaling on the formation of tumors in orthotopic tumor models. Therefore, as we gain a greater understanding not only of the niche of prostate CSCs but also of the factors and cells that contribute to maintaining this environment, further therapeutic strategies can be devised.

5.5 Targeting Stem Cell Differentiation

Theoretically, the process of CSC differentiation is a process that, once understood in more detail, could be targeted. If the prostatic CSCs could be pushed to differentiate into less quiescent, AR-responsive cells then therapies that are efficacious for these cells would be able to eradicate the entire tumor. If the balance of self-renewal and differentiation were tipped entirely to differentiation then presumably the patient could be cured and relapse prevented, due to the loss of all CSCs. This concept has been pondered by several investigators (Lawson and Witte 2007; Mimeault et al. 2007b). Many questions still remain before any sort of therapy could be designed, including (1) what is the definitive identification of the pathways required for self-renewal? (2) can self-renewal pathways be turned off, or can differentiation pathways be turned on sufficiently to drive all of the CSCs to differentiated progeny? (3) if you eradicate one tumor do you still leave behind the propensity for another CSC to be derived in a manner similar to the initial CSC? and, importantly, (4) what will be the impact of derived therapies on normal stem cells?

6 Future Directions

The ability to study and understand the prostatic CSCs is hampered by several factors, including (1) the scarcity of the cells (at most 1% of total cells), (2) a relative lack in culturing techniques that maintain the SC state, (3) adequate techniques that measure SC properties, such as self-renewal. These challenges are currently being addressed and culture techniques have been developed allowing the expansion of these cells, however, it is uncertain if these in vitro expanded cells remain the same as in vivo cells. If the in vitro cultured CSCs do, in fact, retain all

the properties of in vivo CSCs, the challenges investigators currently face due to low numbers of cells will be overcome. Techniques studying self-renewal currently involve serial transplantation of tumor cells. While this demonstrates the retained ability of the cells to initiate a tumor, the method is lengthy, indirect, and does not readily allow for the study of the processes that govern self-renewal.

The cell of origin is also an important topic that needs continued exploration. Does the CSC arise from a normal SC? If so, what is the switch(es) that turns a normal SC into a CSC? Likewise, if the CSC instead arises from a TA cell, what change(s) occurred that allowed the TA to regain the ability to self-renew? Thus, an understanding of not only the pathways governing both self-renewal and differentiation but also the understanding of how this balance is maintained will be important for understanding the etiology of prostate cancer.

With an understanding that prostate cancer develops from a CSC rather than from the transformation of any cell within the prostate, the therapeutic strategies will need to switch focus onto the CSCs specifically. Traditional therapies, for the most part, rely on both the removal of androgen as a stimulus and the ablation of rapidly cycling cells that have a limited ability to repair DNA. Given that the CSC is neither AR^+ nor a fast-cycling cell, new therapies directed at the CSC need to be developed. The ability to do high-throughput screens is also challenging given the low numbers of CSCs that can be isolated using current techniques. Therefore, the understanding of the pathways and biology unique to CSCs that allow for their maintenance is of paramount importance so that more targeted therapies can be derived.

7 List of Abbreviations

RNASEL: ribonuclease L
L HPIN: high-grade pre-neoplastic lesion prostatic intra-epithelial neoplasia,
ADT: androgen deprivation therapy,
GnRH: gonadotropin-releasing agonists,
LH: luteinizing hormone,
CPRC: castrate-resistant prostate cancer,
AR: androgen receptor,
PZ: peripheral zone,
CZ: central zone,
TZ: transition zone,
K: cytokeratin,
CSC: cancer stem cell,
AML: acute myelogenous leukemia,
NOD/SCID: non-obese diabetic and severe combined immunodeficiency,
SC: stem cell,
TA: transit-amplifying,
PSA: prostate-specific antigen,
PAP: prostatic acid phosphatase,

BrdU: bromodeoxyuridine,
SP: side populations,
ABC: ATP-binding cassette,
ABCG2: ABC transporter subfamily member 2,
wnt: wingless-type MMTV site family,
LRP5/6: low density lipoprotein receptor-related protein 5/6,
GSK-3β: glycogen synthase kinase 3-beta,
TCF: T-cell transcription factor,
LEF: lymphoid enhancer-binding factor,
EMT: epithelial-to-mesenchymal transition,
WIF1: wnt inhibitory factor 1,
SFRP: secreted frizzled-related protein,
DKK: Dickkopf,
IHh: Indian hedgehog,
DHh: desert hedgehog,
SHh: sonic hedgehog,
PTCH: patched,
Gli: glioma-associated oncogene homolog,
SMOH: smoothened,
FRZ: frizzled,
HIP: hedgehog-interacting protein,
Fu: fused,
SuFu: suppressor of fused,
ARF: INK4A/ADP ribosylation factor,
ADAM: a disintegrin and metalloproteinase, S; synthesis,
Oct: octamer-binding transcription factor,
Sox: SRY-box 2,
DAPT: *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester,
VEGF: vascular endothelial growth factor,
TGFβ: transforming growth factor beta

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Breast Cancer Stem Cells

Bert Gold and Michael Dean

Abstract One aspect of the analogy between embryogenesis and cancer is the emphasis on rapid cell division and self-renewal from a small number of immortal cells. A key understanding in developmental biology is the concept of determination and its consequences, in the form of lineage totipotency, pluripotency, multipotency, and unipotency. The normal cell fate decision point involves epigenetic mechanisms that are dysregulated in neoplasia. These dysregulated cell proliferation triggers are posited to specifically distinguish tumor-initiating cells from their progeny. Herein we present a review of the embryogenesis of the human breast, with an emphasis on the endocrine and epithelial–mesenchyme interactions required for proper development of tissues in the niche. We expand our conceptualization to include the relationship to the seed and soil hypothesis, and immunoediting theory. We expand on the new paradigm by explaining the relevance of side populations, plating efficiency, and tumor-initiating cells to cancer stem cell theory. Finally, we provide some suggestions for signal transduction pathway interventions, viz., that of the hedgehog/patched pathway, that might make breast cancer more amenable to specific therapeutic interventions.

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1 Introduction

The fertilized oocyte is a totipotent stem cell, capable of giving rise to all the cell types of the embryo and the trophoblast (Gilbert et al., 2006; Kimball, 2003). Even after the first few divisions, embryonic cells give rise to totipotent stem cells, those capable of recreating an entire organism (Seydoux and Braun, 2006). Twinning is often a result of early embryo splitting and subsequent totipotent development (Hall, 2003; Stevenson and Hall, 2006). *Embryonic germ cells* and *embryonic carcinoma cells* are each types of pluripotent stem cells that can be isolated from embryonic or fetal tissue or germ cell tumors. These pluripotent stem cells can be grown in culture to some extent, using feeder layers and growth factors to maintain differentiation capacity. Pluripotent stem cells have a restricted differentiation capacity as compared with totipotent stem cells. There are a plethora of recent reports of both totipotent and pluripotent mammalian stem cells growing in culture: Some of these have been used for mammalian cloning experiments (Jaenisch et al., 2004). Each tissue, as it differentiates, gives rise to the multipotent stem cells of the body (Tsonis, 2007). For example, the hematopoietic stem cell is capable of giving rise to all of the cells in the blood (Lagasse et al., 2000; Till et al., 1964). All stem cells have the property of giving rise to additional stem cells when they divide. This property is self-renewal (Rajaraman et al., 2006). As self-renewal occurs, cells confront a decision point. At this point, cells commit to differentiate and eventually stop dividing, undergoing senescence or apoptosis, or continue dividing (Alberts, 2008; Till et al., 1964). When the decision point results in self-renewal, it permits a nearly immortal lifespan for the stem cell (Schlessinger and Van Zant, 2001). The mechanism underlying the self-renewal decision point is a subject of active investigation (McKenzie et al., 2006; Morrison et al., 1997). Some stem cells, such as

hematopoietic stem cells, maintain relatively rapid division, to produce the large numbers of lymphocytes and red blood cells required by the body (Huang et al., 2007). This is true also of the rapidly cycling cells of the broad band at the middle of the intestinal crypt (Potten, 1991). Other stem cells, such as those in the skin and colon, maintain a slow and constant growth, replenishing the tissue (Alonso and Fuchs, 2003; Bjerknes, 1996), and yet other stem cells in the brain and most other tissues remain quiescent and are only activated when stimulated by tissue damage or hormonal exposure (Schatton and Frank, 2008). When a cell is sufficiently differentiated that it still proliferates, but gives rise to only one cell type, one can speak of it as determined (Spemann, 1918), canalized (Waddington, 1966), or unipotent (Blanpain et al., 2007). An example would be a megakaryocyte that can give rise only to platelets. The only subsequent steps in multicellular organism development are "terminal differentiation" and senescence. Whether or not de-differentiation or trans-differentiation occurs, once cells have progressed through their developmental path, remains a matter of controversy (Leri et al., 2005; Raff, 2003). Stem cell migration is a normal part of mammalian development and is a particular characteristic of the early genital ridge (Gilbert et al., 2006). The regulation of stem cell division in the adult is controlled through epigenetic mechanisms, such as DNA methylation and histone acetylation (Do et al., 2006). Orderly "replenishment" of tissues requires cell division and the capability of repairing some damaged tissues, as in liver regeneration (Michalopoulos, 2007). It is generally acknowledged that the latter requires a certain amount of cellular "reprogramming". A central feature of the involvement of stem cells in cancer is the dysregulation of the epigenetic control of stem cell proliferation (Bapat, 2007; Bengochea et al., 2008; Karpinets and Foy, 2005).

2 Breast Anatomy

In 1840 Astley Paston Cooper published The Anatomy of the Breast (Cooper, 1840). The striking plates in this classical text are based on the author's studies of the breasts of seven previously lactating cadavers through dyed paraffin injection. The illustrations identified numerous structures in the vascular and ductile system and clarified, for the first time, the gross anatomy of the drainage network. The staining techniques were apparently very advanced for that era but crude when compared to modern imaging methods; the wax may have changed and displaced some of the delicate ductile structures of the breast. Moreover, studying a non-lactating breast from a cadaver does not reveal its normal, hormone-mediated growth and development.

The gross anatomy of the human breast shows it to be one of the only organs not fully developed at birth (Lawrence and Lawrence, 1985). The breast changes in size, shape, and function through puberty, pregnancy, and during and after lactation (Creasy et al., 2004). Breast growth and development involve two distinguishable processes: organogenesis (ductile and lobular growth) and lactogenesis (Dontu et al., 2003).

In the developing mammary gland, three cell lineages have been described: myoepithelial cells that form a basal cell layer, ductal epithelial cells, and milk-producing alveolar cells (Dean et al., 2005; Deugnier et al., 2002). Although transplantation studies in mice have demonstrated that most mammary cells have a limited capacity for self-renewal, clonal populations that can recapitulate the entire functional repertoire of the gland have been identified (Smith, 2006; Smith and Boulanger, 2002). In an elegant study, human mammary epithelial cells derived from reduction mammoplasties were used to generate non-adherent spheroids (designated mammospheres) in cell culture and demonstrate the presence of three mammary cell lineages. More importantly, the cells in the mammospheres were clonally derived, providing evidence for a single pluripotent stem cell (Dontu et al., 2003). These same approaches are being used to isolate and characterize breast cancer stem cells (Xiao et al., 2008).

3 Breast Embryology

From the fifth to seventh week of pregnancy, a human fetus develops a mammary ridge, which rises from the axilla to the inguinal region (Ameryckx et al., 2005). By the sixth gestational week, this ridge depresses into the pectoral region, forming primary breast buds (Moore et al., 1993). At birth the main lactiferous ducts are present as well as the nipples and areola (Black et al., 1998).

After puberty, estrogen secretion at each menstrual cycle stimulates proliferation and active growth of breast tissue (Clarke, 2003). Breast development proceeds with growth of the ductile system and the formation of ductile buds (Black et al., 1998). Surrounding fat pads also develop, giving the breast size and shape unrelated to functional capacity (Riordan, 2005).

Estrogen is a potent mammary mitogen that has numerous salutary systemic effects (Ho and Liao, 2002): estradiol, the most active form, decreases risk of coronary artery disease in women between puberty and menopause, a decrease in risk that is not observed in postmenopausal women (Rossouw, 2000). Experimental studies have showed exogenous estrogen can preserve endothelium critical for coronary artery dilation, reduce infarct size, decrease the occurrence of ventricular arrhythmias, and protect against ischemia-reperfusion injury (McCullough et al., 2001; Zhai et al., 2000a,b). Estradiol is also a neuroprotective and neurotrophic factor: it has a positive influence on memory and cognition and may decrease the risk of Alzheimer disease and stroke (Brinton, 2001; Norbury et al., 2003). Finally, estrogen receptor immunostaining has enabled observation of hormonal effects on osteoblasts on the medullary bone surface. Such studies show that estrogen receptors are present in osteogenic cells and suggest that estrogen directly acts on medullary bone osteogenesis (Jilka, 1998). In spite of all these positive activities, exogenous estrogens bring a risk of neoplasia in responsive tissues, probably because of their potent activity as mitogens (Auersperg et al., 2001; Jordan and Morrow, 1999; McLachlan, 2001;

Vona-Davis and Rose, 2007). Obesity has been associated with breast cancer risk (Chlebowski, 2005). Because adipose tissue secretes estrogens, the mechanism through which it acts may be by accumulation of excess estrogen (Deroo and Korach, 2006).

Breast tissue is exquisitely sensitive to the hormonal changes of early pregnancy (Leslie and Lange, 2005) . Many women report breast tenderness as a first sign of pregnancy. The human breast is capable of lactation from 16 weeks post-fertilization, with differing rates of growth and breast development before and after parturition (Lawrence and Lawrence, 1985). During the first trimester of pregnancy, mammary epithelial cells proliferate and duct cells branch in response to estrogen (Sternlicht, 2006). The breast duct epithelium proliferates into the breast fat pads where end buds develop into secretory alveoli in response to human placental lactogen (somatomammotropin), human chorionic gonadotropin, and prolactin (Harrison and Biswas, 1980; Parmar and Cunha, 2004; Riordan, 2005). While progesterone stimulates an increase in the size of the lobes and lobules, somatotropin and ACTH interact with prolactin and progesterone fostering mammogenesis. During the second trimester, there is further enlargement of the duct system and additional growth of the lobules. At approximately 12 weeks, a secretory substance that is similar to colostrum becomes visible in the acini. Subsequent prolactin production from the anterior pituitary together with placental lactogen triggers mammary alveolar differentiation, followed by the glandular secretion of colostrum. The alveoli then become distended with colostrum (Buhimschi, 2004). The dozen or so lactiferous sinuses radiate from the areola, draining into the nipple.

4 Cancer as a Microcosm of Embryogenesis

Most cancer cells divide rapidly and can be grown indefinitely in culture as immortal cells, just as do embryonic stem cells. Embryonal carcinomas and teratocarcinomas are tumors derived from embryonic cells and can differentiate and give rise to cells of many lineages. In 1976 Beatrice Mintz and Ralph Brinster independently showed teratocarcinomas could give rise to normal chimeric mice (Brinster, 1976; Mintz and Illmensee, 1975).

4.1 Soil and Seed

In 1889, the English physician Stephen Paget introduced the "soil and seed" hypothesis of metastasis to English-speaking medicine, by crediting the idea to Fuchs (Fuchs, 1882). In Paget's study of 735 fatal cases of breast cancer, he concludes that the distribution of metastases cannot be due to chance alone and that different tissues provide optimal conditions for the growth of specific cancers. He noticed that patients with primary breast cancers had secondary

tumors that developed preferentially in regional lymph nodes, bone marrow, lung, and liver (Paget, 1889). In the "soil and seed" metaphor, the "soil" refers to the secondary site of tumor development, and perhaps the chemical signals produced in the microenvironment at the potential site of metastasis (Langley and Fidler, 2007; Strieter, 2001). The "seed" is the ostensible stem cell or tumor-initiating cell from the primary tumor (Chung et al., 2005). Genetic variations that affect signaling molecules in the metastatic microenvironment can impact the "soil" (Crawford and Hunter, 2006; Eccles and Welch, 2007). Overexpression of cellular migration factors could encourage a faster movement or more rapid growth of tumor cells and could challenge the capacity of immunosurveillance to keep a tumor in check. Upregulation of cell surface receptors on tumor cells could provide a propitious key to unlock a fertile new "soil" for them. Mutations that affect the autocrine and paracrine signaling, in for example chemokine receptors and their effector molecules, could play an important role on tumor growth exacerbation or inhibition. Relief of immune inhibition is known to play an important role in immunosurveillance and could be responsible for a significant amount of tumor escape. Variations that augment inhibitory factors could have a protective effect by decreasing the rate of tumorigenesis.

In a variation of this idea, called the "homing" hypothesis, a secondary signal secreted by cells at the future metastatic sites "calls" the tumor cells and permits them to proliferate there (Hewitt et al., 2000; Stetler-Stevenson, 2001). In this hypothesis, the "seed" produces cell surface receptors able to recognize the site demarcated by the "soil". Although the mechanisms of tissue specificity remain obscure, researchers have focused on small messenger molecules as attractants and larger cell surface receptors guiding the tumor-initiating cells or "seeds". Muller (Muller et al., 2001) and Murphy (Murphy, 2001) have each focused on chemokines and chemokine receptors as viable candidates for "soil and seed" signaling. Murphy (Murphy, 2001) specifically proposes a "spatial and temporal code" made up of specific combinations of such molecules, and others being responsible for neovascularization, metastasis, and immunosurveillance avoidance.

Chemokines and their receptors have been implicated in three distinct stages of neoplasia: transformation, tumor development, and metastasis. Expression of specific receptors on KSHV-infected B-lymphocytes and the expression of specific receptors in HIV patients, such as CCR5 or CXCR4, are sufficient to dictate the future course of their respective diseases. Other cancers may involve specific chemokine receptor expression (Strieter, 2001).

4.2 Metastasis

Metastasis is the most troublesome property of tumor cells (Barnhart and Simon, 2007). The majority of cancer fatalities are due to the effect of the spread

of the initial tumor to other sites (Lou and Dean, 2007). Basal cell carcinomas, although they can be quite invasive, virtually never metastasize and are rarely fatal. Stem cells, especially certain cells of the neural crest, possess the ability to migrate through the developing embryo. The neural crest gives rise to the precursors of melanoma, neuroblastoma, and small cell lung cancers, each of which is a highly metastatic tumor (Kasemeier-Kulesa et al., 2008). Is the metastatic tendency of tumor cells related to an innate property of the cancer stem cells to migrate (Ward and Dirks, 2007)? If so, then the further characterization of germ cell migration could lead to new insights into metastasis. Anticancer stem cell therapies might find their best application in the restriction of metastasis. If metastasis could be prevented, even if the primary tumor remains intact, then the patient might still experience a substantial increase in survival time.

4.3 Elimination, Equilibrium, and Escape

In 2001, Shankaran et al. proposed some modifications to the then predominant theory of immunosurveillance (Shankaran et al., 2001). Later, these were refined into a hypothesis of "immunoediting" (Dunn et al., 2004a,b). The cancer immunoediting hypothesis proposes that cell-mediated immunosurveillance as conventionally described takes place, yet envisions three different resulting outcomes. These are described in the three Es as: elimination wherein the immune system can eliminate cancer; equilibrium, wherein immunosurveillance holds an incipient malignancy in check; or escape, wherein the cancer can escape from the immunosurveillance, often metastasizing. The authors prefer their paradigm to the use of the older term immunosurveillance, essentially because their paradigm anticipates opportunities to intervene in each of the three predicted outcomes of "immunoediting". Cancers that inexplicably go into remission for years provided early evidence for equilibrium. In addition, there have been a few case reports of organ transplants that have transferred undetected dormant tumors to the recipients. These provide additional evidence for "equilibrium". In this model, chemical carcinogenesis is explained as the escape of small, latent growths from equilibrium. Evolutionarily, it is thought that the immune system has not evolved specifically to handle cancers, as these are mainly diseases of the post-reproductive elderly. Thus no selection pressure, per se, has been exerted on the immune responses to eliminate post-reproductively acquired cell division maladies. The immunoediting hypothesis states that the transformed cell may look like a foreign cell to the cell-mediated immune response. Cancer cells can then reduce their antigenicity by changing the epitopes they show to the immune system so that they more closely resemble those presented by normal tissue. If tumor cells are successful in this "Trojan Horse" strategy, they enable the final outcome of the immunoediting theory: escape and metastasis.

5 Central Importance of the Stroma

Luminal epithelial cells interact with a surrounding microenvironment (Sternlicht, 2006). In part, these interactions direct normal mammary gland development. Altering luminal epithelial cell interaction with the extracellular matrix and local microenvironment might induce abnormal intracellular signaling pathways that affect the development and progression of breast tumors (Gupta and Massague, 2006). Mina Bissell and her group at Lawrence Berkeley Laboratory have been studying these kinds of interactions for the past 20+ years (for review see LaBarge et al., 2007). A central signal pathway for mammary gland development and breast cancer progression involves the expression of estrogen receptors (Novaro et al., 2004). In a study using cultured nonmalignant mammary epithelial cells, the basement membrane molecules, laminin-1 and collagen-IV, were found to be involved in maintenance of estrogen receptor alpha expression (Novaro et al., 2003). Other workers found that this response could be interfered through the disruption of cell-extracellular matrix adhesion. Phenotypically normal mammary epithelial cells have been used to dissect the promoter region of the ER alpha receptor involved in response to the basement membrane. A malignant cell line sharing a common lineage with normal mammary cells provide the insight that overexpression of ER alpha accompanied unresponsiveness to normal basement membrane regulation found in those malignant cells. One interpretation of these data is that cross-talk between different signaling pathways is a requirement in the constitution or proper functional tissue organization and when this cell–cell interaction goes awry, the malignant phenotype may result.

Normal tissue homeostasis is maintained by dynamic interactions between epithelial cells and their microenvironment. As tissue becomes cancerous, there are reciprocal interactions between neoplastic cells, adjacent normal cells such as stroma and endothelium, and their microenvironments. The current dominant paradigm wherein multiple genetic lesions provide both the impetus for, and the Achilles heel of, cancer might be inadequate to understand cancer as a disease process.

6 Breast Cancer Stem Cells

A University of Michigan group recently identified a small population of cancer stem cells in breast tumors that has changed the way many scientists view cancer (Al-Hajj et al., 2003; Hemmati et al., 2003). These cancer stem cells represent only 1% of the tumor and were the only cells in the tumor capable of transplanting the tumor into nude mice. This suggests that the terms cancer stem cells and tumor-initiating cells are functionally synonymous. Additional studies have presented data that long-established cell lines, even HeLa cells, contain a minor population of cells with some of the same tumor-initiating properties as

stem cells (Hirschmann-Jax et al., 2004; Kondo et al., 2004). Many researchers now suspect that all cancers are composed of a mixture of stem cells and proliferative cells with a limited life span.

The implications of this concept are far reaching. The regrowth of many cancers following chemotherapy could result from the survival of cancer stem cells. This is paralleled in the body with the regrowth of hair due to the survival of hair follicles and the recovery of blood cells due to the survival of hematopoietic stem cells. Can these results be extrapolated to most or all solid tumors? Are there therapeutic approaches targeting these cancer stem cells with application to a wide array of cancers? These are critical questions remaining to be addressed in the cancer stem cell field.

Researchers have known for decades that there exist a proportion of cells in a tumor capable of surviving radiation treatment and cytotoxic drug exposure (Thomlinson and Gray, 1955). These cells are capable of DNA repair and can survive and reproduce under hypoxic conditions (Teicher, 1994; Thomlinson and Gray, 1955). Stem cells must also survive many genetic insults in the life of the individual and express drug transporters and DNA repair systems. Stem cells are necessarily refractory to programmed cell death and can be quiescent for long periods of time, all properties that would allow a cancer cell to resist standard therapeutic approaches (Kim et al., 2002; Scharenberg et al., 2002; Zhou et al., 2001).

7 Multistep Carcinogenesis and DNA Repair

One of the most important discoveries in cancer research has been the elucidation of the multistep nature of cancer and the development of experimental systems that allowed the nature of carcinogenic agents to be explored (Vogelstein and Kinzler, 1993). In 1941 Peyton Rous demonstrated that when rabbit skin was exposed to mutagens the resultant tumors would not form unless there was a subsequent wounding at the site (Rous and Kidd, 1941; Shubik, 2002). This later event could be carried out long after the original exposure. Subsequently the terms initiator and promoter were coined (Rous, 1966). Initiators are agents that animals had to be exposed to initially to develop a tumor. While many exposures with a tumor initiator could be shown to cause cancer, the typical experiment involved a single exposure to the initiator and multiple subsequent exposures with a tumor promoter (Kim et al., 2002). The promoter had to be given multiple times and within a fairly constrained window.

The mechanism of action of tumor initiators was the first to be understood. They could all be shown to be agents damaging DNA and causing mutations (Alberts, 2008). Tumor promoters were more elusive and included agents such as phorbol esters and mineral oils. In addition, wounding could also be shown to cause tumor promotion (Hennings and Boutwell, 1970). Eventually some tumor promoters were shown to activate cells via the protein kinase C pathway (Castagna et al., 1982).

Tumor promoters can then be thought of as agents that disrupt the tissue and activate stem cells, while tumor initiators damage DNA and mutate specific genes. The only difference between the experimental animal models of tumorigenesis and the human situation is the fact that human cancers involve a chronic exposure to tissue-damaging agents and mutagens (Tennant, 1999). Most of the environmental agents that lead to stem cell activation would be classified as promoters, non-DNA reactive agents that enhance the tumorigenicity of initiators (mutagens). Some environmental agents, such as tobacco smoke and UV irradiation, contribute to both initiation and promotion as they stimulate tissue proliferation and damage DNA.

In light of the identification of a small population of cancer stem cells, the initiation and promotion scheme can be updated. Cancer can be envisioned to be caused by a combination of agents disrupting tissue architecture and activating stem cells, and agents that damage and mutate DNA. This paradigm explains the role of known agents implicated as causing cancer and focuses the problem on a subset of abnormal cells that could be specifically targeted, resulting in more effective therapies.

8 The Origins of the Tumor-Initiating Cell

Cancer stem cells can arise from two potential pathways. A multipotent stem cell with abnormal growth control can give rise to a cancer stem cell (Reya et al., 2001). Alternatively, a progenitor cell or more committed cell could acquire the self-renewal capabilities and become a cancer stem cell (Shipitsin and Polyak, 2008). There is evidence for both mechanisms, and some have argued for a dynamic process within the tumor. Transfection studies where activated oncogenes can transform murine fibroblasts document that non-stem cells can be converted into cancer (stem) cells. Evidence has also been found for the fusion of stem cells with tissue cells, creating cells with self-renewal capacity (Houghton et al., 2004).

From 2001 to 2008, Robert A. Weinberg and colleagues (Elenbaas et al., 2001; Ince et al., 2007; Zimonjic et al., 2001) were able to demonstrate that sequential introduction of three cancer-associated genes into human mammary epithelial cells (HMECs) renders these cells tumorigenic. Genetic complexity of breast cancer cells had heretofore made it difficult to identify the specific genetic alterations that are required for the switch from a normal cell to a tumor cell. In this work, Weinberg's group first focused on creating a human xenograft mammary cell model (Kuperwasser et al., 2004) and later on specific gene transformations using SV40 large-T antigen, telomerase hTERT, and oncogenic H-rasV12, each of which interfere with essential regulatory pathways governing cellular growth and survival. Sequential introduction of these three genes into HMECs results in cells that display the typical properties of malignant cells in culture, and form aggressive tumors when transplanted into mice.

As a follow-up these investigators sought to characterize c-myc oncogene amplification in the resulting HMEC tumors (Ben-Porath et al., 2008). Because this gene frequently mutated in breast cancer, amplification is taken to signify initiation of a process of additional events that recapitulate further genetic alterations typically found in the spontaneous development of breast cancer in patients.

9 Activation of Stem Cells and Cancer

Most stem cells in the body remain in a dormant state. These cells are surrounded by other, differentiated cells within the tissue microenvironment often described as a “niche”. The cells of the niche regulate the stem cells via cell–cell contacts, interactions with the extracellular matrix, and secretion of inhibitory factors. The disruption of the niche microenvironment, through infection, inflammation, tissue damage, or chemical assault, can activate the division of the stem cells (see Fig. 1). The activated stem cell gives rise to additional stem cells as well as cells committed to differentiate. These new cells repair the damaged area of tissue, and the stem cells return to their quiescent state. Virtually all of the agents described to confer a risk for cancer also result in tissue alteration (and therefore activation of stem cells) including radiation, wounding, chemical damage, infectious agents, and inflammation.

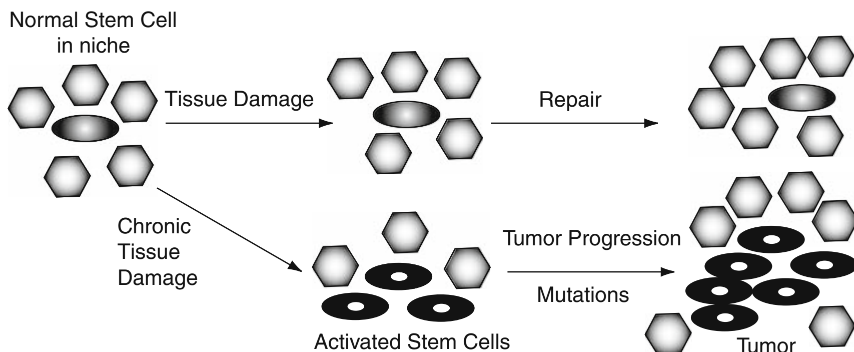

Fig. 1 Activation of stem cells and tumor progression. Stem cells are quiescent in the niche. Upon tissue damage they divide and repair the damage. However, chronic tissue damage leads to continually divided (activated) stem cells that are the target for later mutagenic events that create a cancer stem cell and a tumor

Cancer can be thought of as a disease resulting from the abnormal growth of stem cells, resulting from chronic activation of stem cells (caused by disruption of the niche) and leading to the long-term proliferation of the stem cells. Chronically dividing stem cells are a target for additional mutagenic agents

resulting in genetic damage to the cell (mutation of tumor suppressor genes and activation of oncogenes). This disruption of the niche and subsequent stem cell activation could occur by hormonal stimulation, tissue damage caused by inflammation, radiation, chemicals, or infections, or inactivation of certain tumor suppressor genes. The abnormally dividing stem cell could be subject to additional genetic events leading to autonomous growth, the loss of cell cycle regulation, and resistance to apoptosis—all well understood properties of cancer cells (Beachy et al., 2004)

10 Tumor Suppressor Genes and Cancer-Initiating Cells

In 1993 Alfred G. Knudson, Jr. proposed that the action of tumor suppressor genes could be explained by their effects on tissue stem cells (Knudson, 1993) (see Fig. 2). He distinguished those tumors deriving from embryonic tissue, from tissues under hormonal control, or renewal tissues, like the skin and gut. The first direct evidence for the presence of a stem cell in a cancer came from the work of John Dick. In 1994 his group demonstrated the presence of cancerous stem cells in acute lymphocytic leukemia by cloning such cells and documenting their self-renewing capacity, the critical property of all stem cells (Lapidot et al., 1994). The identification of the *Patched* (*PTCH*) gene as a tumor suppressor gene directly connected early development to tumorigenesis (Hahn et al., 1996a; Hahn et al., 1996b; Johnson et al., 1996). The PTCH protein is a membrane

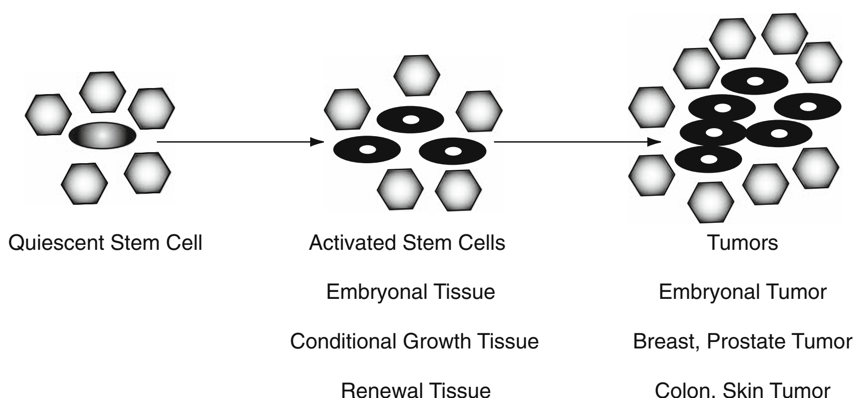

Fig. 2 Stem cell activation and cancer. Three major types of tumors are recognized. Embryonal tumors derive from embryonal tissue, in which the stem cells are in a highly proliferative state. These cells can directly progress to tumorigenicity with the appropriate mutations. Conditional growth tissues such as the breast and prostate are under hormonal control. During hormonal stimulation, such as during puberty, the stem cells are undergoing dramatic expansion and the stem cells are susceptible to mutagenic events. Renewal tissues undergo a slow regular division. The mutation of genes such as APC in the colon and PTCH in the skin produces activated stem cells and the potential for progression

protein with 12 membrane spanning segments and is the receptor for the Hedgehog (HH) family of signaling molecules. PTCH and the HHs play a central role in the cell fate and patterning of early embryonic cells. Alteration in the growth regulation of stem cells was invoked to explain the role of the *PTCH* gene in causing basal cell carcinomas in patients with nevoid basal cell carcinoma syndrome (Dean, 1997).

11 Stem Cell Activation and Specific Cancers

While a model of a small population of self-renewing cells as the key to all cancers is an attractive idea, can the model be extended to the wide variety of tumor types and specific agents implicated in causing these tumors? Three distinct types of cancers have been described—embryonic, conditional growth, and renewal (Knudson, 1993). Embryonic cancers derive from rapidly dividing embryonic tissue and therefore contain a population of actively dividing stem cells. The prototype embryonic cancer is retinoblastoma (Knudson, 1971), but Wilm's tumor, Ewing's sarcoma, childhood bone and brain cancers each fall under this rubric. Retinoblastoma arises in embryonic cells in the developing eye, known as retinoblasts. These cells are highly proliferative and are naturally activated stem cells. The mutation or loss of the *RB1* gene transforms these embryonic stem cells into cancer stem cells. These cells would be expected to have lost the response to growth regulatory signals shutting down the stem cell, once the development of the eye was complete. Other childhood cancers could involve multipotent stem cells in other tissues suffering genetic damage during development. These cancers require the fewest number of genetic events, because the target cell is a fully activated stem cell.

Stem cells can also be activated during the normal process of expansion of certain tissues due to the action of hormones, particularly during puberty (conditional growth tissues) (Knudson, 1993). Examples would be the breast and prostate, which undergo dramatic expansion and growth during puberty under the control of estrogen, testosterone, and other hormones (Trichopoulos et al., 2005). Activated stem cells in the breast would be the target cell for breast cancer. Inactivation of specific tumor suppressor genes, like *TP53,* would transform the breast tissue stem cells into unregulated cells, initially resulting in pre-malignant lesions. There is good evidence that p53 haploinsufficiency accelerates cancer onset, perhaps by diminishing DNA repair, thereby facilitating mutation of activated stem cells. These uncontrolled stem cells would be the target for additional events leading to the progression of the pre-malignant lesion into a fully malignant tumor.

Consistent with this model, the major risk factors for breast cancer involve hormonal and reproductive variables (Gail et al., 1989). Women with an early onset of puberty have a higher rate of breast and ovarian cancers than those with later menarche. Pregnancies, especially those starting at a relatively

younger age, decrease cancer risk. These factors influence either the number or activation of breast stem cells. Several drugs able to decrease cancer risk and/or cancer reoccurrence have been developed. These include agents reducing the production of estrogen or blocking its action on cells. Similarly the removal of the ovaries reduces cancer risk in those with an extensive family history of breast and ovarian cancer (Kauff et al., 2002). Mutations in the *BRCA1* and *BRCA2* genes dramatically increase the risk of breast cancer. However, unlike many other tumor suppressor genes, *BRCA1* or *BRCA2* mutations are not commonly found in sporadic breast tumors. The BRCA1 and BRCA2 proteins play a role in the DNA repair process. These mutations can be thought of as increasing the probability of genetic events associated with tumor progression. Since these genes are not in the main pathway leading to the breast cancer, they are not frequently mutated in sporadic tumors, but do increase an individual's risk of disease, when mutated.

In tissues such as the colon or skin, undergoing a constant level of renewal of the cells, the stem cells are dividing at a slow but constant rate. In the absence of disruption of these tissues, the risk of cancer is low. However, activation of stem cells in renewal tissues can occur by inflammation or by tissue damage caused by radiation, mutagens, and irritants (Rakoff-Nahoum, 2006; Trosko, 2005; Trosko and Tai, 2006). Chronic tissue damage would cause an increased rate of division of the renewal tissue stem cells and increase the available number of target cells for transformation. Colon cancer provides one of the best examples of the influence of inflammation on cancer. Inflammatory diseases such as Crohn disease and inflammatory bowel disease result in dramatically increased risk for colon cancer (Eaden, 2004). Patients with mutations in the *APC* gene have familial adenomatous polyposis coli (FAP) syndrome and suffer from large numbers of colon polyps (Groden et al., 1991 Nishisho et al., 1991). Polyps are pre-malignant lesions and FAP subjects have an elevated risk for colon cancer (Gardner, 1962). Individuals with FAP, as well as the mice with mutations in *Apc*, have an increased cellular proliferation compartment in the colon (Deschner and Lipkin, 1975; Moser et al., 1992; Su et al., 1992). The effect of *APC* mutations has been proposed to be to increase the proliferation of colonic stem cells (Knudson, 1993). Consistent with this model, the *APC* gene is mutated in the vast majority of sporadic colon tumors (Kinzler and Vogelstein, 1996). Additional downstream events in colon cancer are very well characterized and include mutations in *P53*, *RAS*, and other genes (Kinzler and Vogelstein, 1996).

12 SP Cells in Tumors and Cell Lines

Once it was recognized that stem cells were predominantly found in the Side Population (SP) fraction, it became possible to sort and purify stem cells from virtually any population of cells or tissues. SP cells were identified in 15 of 23 neuroblastoma samples and in neuroblastoma, breast cancer, lung cancer, and

glioblastoma cell lines (Hirschmann-Jax et al., 2004). Furthermore, analysis of several cell lines that had been maintained in culture for long periods of time demonstrated a small population of SP cells. In the rat glioma C6 cell line, a population of cells containing SP characteristics was isolated from a non-SP population. Through the use of growth factors these investigators could maintain these cells in culture, and show that only the SP cells gave rise to both populations, and produced cells with both neuronal and glial markers that were tumorigenic in mice (Kondo et al., 2004). This latter study provided strong evidence that in this cell line, the SP population reflected a population with a capacity for self-renewal and limited maturation. However, this isolation approach is imperfect as the SP compartment is composed of stem and non-stem cells, and some stem cells are not in the SP fraction (Zhou et al., 2002). For example, non-stem cell tumor cells often express *ABCG2* and *ABCB1*. These genes are highly expressed in drug-resistant cells, and histopathologic studies have reported increased expression of the ABCB1 transporter in more differentiated tumors (Mizoguchi et al., 1990; Nishiyama et al., 1993). In addition, in a variety of cell lines, differentiating agents induce expression of ABCB1, inhibit cell growth, and increase the expression of markers of maturation (Bates et al., 1989; Mickley et al., 1989).

Additional limitations exist in using cancer cell lines cultured in vitro to study stem cell biology and drug resistance. Although SP cells and cells with stem cell properties have been reported in cultured cell lines, it is difficult to reconcile the hypothesis that only a small fraction of cells in culture possess the ability to proliferate and self-renew with the rapid doubling time of cells in culture. Current paradigms envision a small stem cell compartment possessing cells with the capacity for perpetual self-renewal existing alongside a much larger proliferative compartment whose cells have a finite ability to proliferate before presumably arresting and/or undergoing apoptosis. These paradigms can explain the low cloning efficiency of most cell lines, their inefficiency at colony formation in soft agar, and their limited tumorigenicity. But none can explain how the stem cells remain a constant fraction of the total population, if indeed they do. Any proposal will require stem cells to divide slowly, and must recognize that in a cell line derived from a solid tumor the number of cells undergoing apoptosis is relatively small. One possibility is that there is an interchange of cells between a proliferative compartment and the stem cell pool. That such an interchange might occur is not improbable since the cell line almost certainly originated from a stem cell with a proliferative advantage.

13 Major Cancers and Risk Factors

Environmental risk factors have been identified for most of the most common cancers. These risk factors can be classified by their potential role in either activating stem cells or mutating target genes.

13.1 Cancer Therapy "Causes" Cancers

If most solid tumors are composed of a minor population of self-renewing (stem) cells and a large fraction of non-renewing cells, cancer therapy failure following radiation and chemotherapy treatments is not the result of a rare cell evolving from within the tumor, but the regrowth of the cancer stem cells. Of course, tumor stem cells could accumulate genetic changes rendering them even more drug resistant, radiation resistant, or aneuploid. Because cures are achieved for many types of cancer, the cancer stem cells must be eliminated by a given therapeutic strategy. Mature, committed stroma in the tumor microenvironment are likely to play a role in supporting or stimulating the stem cells, forming a "tumor niche". The rapid regression of the tumor could lead to disruption of the tumor niche and the elimination of the cancer stem cells. Immune surveillance is clearly important in many cancers (Nakachi et al., 2004), and reducing the mass of the tumor may allow the immune system to efficiently recognize the remaining cells.

Targeted therapies directly suppressing or killing tumor stem cells may synergize with established therapies to provide increased efficacy. Angiogenesis is likely to be critical to provide blood supply to the tumor stem cells, and strategies to inhibit the development of blood vessels are likely to be effective (Folkman, 2002).

One of the protective mechanisms of stem cells against toxins is the expression of one or more ATP-binding cassette (ABC) efflux transporters. These pumps protect stem cells from xenobiotic toxins (Gottesman et al., 2002). The *ABCG2* and *ABCB1/MDR1* genes are expressed in the majority of stem cells and in most tumor stem cells (Kim et al., 2002; Scharenberg et al., 2002; Zhou et al., 2001). These transporters can efflux fluorescent dyes such as rhodamine and Hoecht 33342, and this property allows stem cells to be separated from non-stem cells on a cell sorter (Goodell et al., 1996). The combined use of chemotherapy drugs and ABC transporter inhibitors could be used to specifically target cancer stem cells (Dean and Annilo, 2005). There are highly specific inhibitors of ABCB1 in clinical use and ABCG2 inhibitors in development (Henrich et al., 2006). Transporter inhibition therapies are likely to have toxic effects on the patient's normal stem cells, and both ABCG2 and ABCB1 play a role in the blood–brain barrier. Therefore, this approach would have to be carefully adjusted to avoid excessive toxicity.

14 How the Stem Cell Paradigm Suggests New Approaches

Another approach to inhibiting cancer stem cells is to target the proteins essential for the growth and maintenance of stem cells. Because of the fundamental research in *Drosophila*, mice, *C. elegans*, zebrafish, and other developmental systems, a tremendous amount is known about the growth regulatory

pathways functioning in embryonic cells (Nusslein-Volhard and Wieschaus, 1980). One pathway, controlled by the Hedgehog (HH) and WNT signaling molecules, contains several genes functioning as either tumor suppressor genes or oncogenes (Dean, 1997). For example *Patched* (*PTCH*) is the receptor for HH molecules and *PTCH* is mutated in patients with nevoid basal cell carcinoma syndrome (Chidambaram et al., 1996; Hahn et al., 1996a; Johnson et al., 1996). The *PTCH* gene is also mutated in virtually all sporadic basal cell carcinomas and in some medulloblastomas, rhabdomyomas, and rhabdomyosarcomas (Bale and Yu, 2001; Gailani et al., 1996; Tostar, et al., 2006). The mammalian *HH* genes (*IHH*, *SHH*, *DHH*) are overexpressed in a large number of cancers including small cell lung, pancreas, gastric, breast, and prostate (Berman et al., 2003; Karhadkar et al., 2004; Thayer et al., 2003; Watkins et al., 2003). *HH* ligand overexpression and *PTCH* mutation both have the effect of constitutive expression of *smoothened* (*SMO*), a G-protein-coupled receptor family protein, a key signaling protein in the pathway. Constitutive *HH* expression could be an important component to the stem cell activation in many cancers and therefore represents an attractive target for cancer therapy.

Cyclopamine is a compound discovered in the Corn Lily (*Veratrum californicum*), a plant teratogenic to sheep (James et al., 2004). Cyclopamine binds to and inhibits the SMO protein and suppresses the growth of cells and tumors with activated HH signaling (Chen et al., 2002). Human prostate tumor cell lines grown as xenografts in mice were eliminated following 21 days of treatment with cyclopamine (Karhadkar et al., 2004), and UV-induced basal cell carcinomas were suppressed in mice given low levels of cyclopamine in their drinking water (Athar et al., 2004). Recently it has been demonstrated that vitamin D3 is a critical signaling molecule between PTCH and SMO. PTCH normally secretes vitamin D3 and this molecule inhibits SMO on that cell as well as adjacent cells (Bijlsma et al., 2006). HHs inhibit this secretion and cause a release from repression. Cyclopamine competes for the binding of vitamin D3 on SMO and so appears to act in a similar manner. It is likely that vitamin D3 and/or other steroidal analogues could have a similar effect and be candidate anticancer compound.

Other pathways critical to embryonic development and potentially important in cancer have also been described and include the WNT and NOTCH pathways. A number of experimental inhibitors of these pathways have been developed. These pathways are also the subject of drug development for a number of conditions and one example is the drug MK0752, which is in clinical trials for the treatment of acute T-cell lymphoblastic leukemia, myelogenous leukemia, chronic lymphocytic leukemia, and myelodysplastic syndrome. Gamma-secretase is required for the maturation of the NOTCH protein, and γ-secretase inhibitors have been developed for a number of pathological conditions. In a recent study, one such gamma secretase inhibitor was effective in the inhibition of stem-like cells in embryonal brain tumors (Fan et al., 2006).

15 Future Research

The identification of cancer stem cells in solid tumors has important implications for basic cancer research. Most analyses of tumors such as gene expression, microarray, proteomic, and many phenotypic assays have been performed on whole tumors and have not revealed data on the small fraction of tumor stem cells. In addition, screens for cancer cytotoxic drugs have involved cell cultures treated over short time periods (Alley et al., 1988). Drugs specifically targeting cancer stem cells may display modest activity in short-term proliferation assays and be rejected for further follow-up study in animals or humans.

Several important questions remain from the current data. Are current markers for cancer stem cells adequate? Do the side population cells isolated from cell lines (Hirschmann-Jax et al., 2004; Kondo et al., 2004) bear a relationship to cancer stem cells? In principle in any permanent cell line there must be self-renewing cell population. If the characterization of the SP cells in cell lines could be applied to cancer stem cells, this could advance understanding rapidly. One property of cancer cells is the ability, like stem cells, to grow in soft agar cultures (Hamburger and Salmon, 1977). It has been found that only a fraction of cells in a tumor cell culture can form a colony in soft agar. Are the cells forming soft agar colonies cancer stem cells? This would be a logical conclusion from the information at hand. It is known that the clonogenicity varies substantially between different tumor cell lines. If clonogenicity is related to self-renewing cells in the culture then assays based on colony formation may be useful for screening for stem-cell-targeting therapies. Such assays would be more time consuming and have a lower throughput, but might in the end prove more informative.

16 Conclusions

The identification of cancer stem cells in certain solid tumors has created considerable excitement in the field and generated new research possibilities. If these results can be extended to most or all cancer cell types, a considerable advancement in understanding will be achieved. Separating the cancer process into a stem cell activation phase and a tumor progression phase allows an understanding of how the myriad cancer causing agents can have their effect on specific tissues. Research efforts directed to understand the growth requirements of tumor stem cells as well identify tumor stem cell antigens could lead to new targeted approaches.

The isolation and characterization of cancer stem cells from other tissues will be a great aid in cancer diagnostics, cancer prevention, and therapeutics. Normal stem cell-based approaches are being intensively developed as an aid in replacing damaged cells and tissues in the body. The insight from the growth and characterization of normal stem cells will aid in the understanding of cancer stem cells and in new therapeutic approaches.

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Stem Cells and Lung Cancer

Adam Yagui-Beltrán, Biao He, and David M. Jablons

Abstract Lung cancer with its more than 1 million deaths per year is the leading cause of cancer mortality for both men and women worldwide. Despite recent advances in current treatment modalities, overall survival rates have hardly improved. Overall 5-year lung cancer survival rate is approximately 15% in the USA and these numbers are much lower in the developing world. The intrinsic resistance shown by stem/progenitor cells following traditional chemotherapy leads to disease recurrence and decreased patient survival and is a major clinical challenge to overcome. Populations of cancer stem cells (CSCs) have been found and characterized in multiple malignancies such as many hematological, breast, colorectal, brain, pancreatic, and maxillofacial cancers; however, this has not fully happened yet in human lung cancer, making such a task a paramount necessity. In this chapter we explore the roles of the main developmental signaling pathways in lung organogenesis and maintenance, together with the issue of homeostatic pulmonary stem cells within specific 'niches' in the bronchopulmonary tree. We explain how aberrations inflicted in many of the components of this complex homeostatic machine can lead to the formation of lung cancer stem cells with accumulated permanent mutations that allow them to repopulate their tumors rendering these lesions resistant to traditional cytotoxic treatments, resulting in dismal prognosis and poor survival rates. The aim, of course is to ultimately integrate the knowledge of these mechanisms into tangible tools that can be eventually translated into novel therapies for lung cancer.

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1 Introduction

Lung cancer is the leading cause of cancer mortality for both men and women worldwide (Parkin et al., 2005). Overall 5-year survival rates for this disease have only marginally changed over the last few decades, with current 5-year survivals being around 15% in the USA (Jemal et al., 2007) and much lower in developing countries. The intrinsic resistance of stem and progenitor cells following therapy results in disease recurrence and decreased patient survival and represents a challenge to successful management. The study of cancer-initiating tumorigenic or 'cancer stem cells' has been gaining importance in the scientific and medical community as the number of cancers containing a 'cancer stem cell' component continues to increase (Bonnet and Dick, 1997; O'Brien et al., 2007; Ricci-Vitiani et al. 2007). Populations of cancer stem cells have been characterized in various malignancies such as many blood, breast, colorectal, brain, pancreatic, and maxillofacial cancers (Bonnet and Dick, 1997; O'Brien et al., 2007; Ricci-Vitiani et al. 2007; Lapidot et al., 1994; Al-Hajj et al., 2003; Singh et al., 2004; Li et al., 2007; Prince et al., 2007; Dalerba et al., 2007), but not in human lung, emphasizing the importance to do so. Targeting of a unique and phenotypically defined pulmonary cancer stem cell population exhibiting discrete therapeutic vulnerabilities will allow better disease-free intervals and survival rates (Reya et al., 2001; Pardal et al., 2003; Wicha et al., 2006). The field of pulmonary stem cell biology is growing and will result in the steady identification of multi-potent, self-renewing, and proliferative progenitor cell populations throughout the bronchopulmonary tree (Rawlins and Hogan, 2006; Berns, 2005). These cells give rise to both transiently amplifying (TA) and terminally differentiated (TD) cells, which are important for tissue maintenance (Hong et al., 2001; Benitah et al., 2005). In leukemia, it has been shown that partially committed cells, which are normally responsible for tissue maintenance after trauma, may undergo transformation via mutations resulting in the selective expression of genes that accentuate and perpetuate these cells' self-renewal capabilities (Bonnet and Dick, 1997; Al-Hajj et al., 2003; Blair et al., 1997; Passegue et al., 2003; Kondo et al., 2004; Patrawala et al., 2005). Bearing this in mind it is valid to view stem cells as protumorigenic. It has been proposed that the accumulation of oncogenic events may 'lock' activated stem cells in a perpetual aberrant state, which converts normal homeostatic stem cells into 'cancer stem cells' (Beachy et al., 2004; Fig. 2). Because many developmental signaling pathways drive these cells into neoplasia we are required to understand in some detail their intricacies.

2 Embryological Signaling Pathways in Lung Cancer

Liu et al. compared the molecular signatures of different human lung cancers against the changing expression profiles of mouse orthologs at different stages of mouse lung development: normal human lung samples were histologically most resembling of late mouse lung embryological stages; human small cell lung cancer (SCLC), which is the most aggressive malignant subtype of lung cancer, appeared first. Squamous carcinoma followed and then adenocarcinomas, which have the best 5-year survival rates. The observation that the earlier stage of mouse lung development to which a human lung tumor resembled was linked with worse survival rates suggested that abnormally activated embryological cell signaling pathways are important in lung carcinogenesis (Liu et al., 2006). It is fascinating that these experiments justly support Rudolf Virchow's old hypothesis of '*Omnis cellula e cellula*' ('Every cell is originated from another mother-like cell'), which proposes that embryonic cells are the originators of cancer (Virchow, 1858). As we continue to understand in detail the repercussions of deregulation in key embryonic signaling pathways, we begin to dissect their intricate relationships with stem cells and malignancy (Reya et al., 2001; Pardal et al., 2003; Wicha et al., 2006). In order for an organism to develop, its cells need to be able to proliferate and to follow specific fates of cellular differentiation in a tightly controlled temporospatial manner, to ultimately ensemble and organize into functioning tissues and organs. Cell fate specification and complex gene regulatory networks (GRN) control development (Davidson and Erwin, 2006). Cellular phenotypes are the result of patterns of gene expression. Intrinsic transcription factors released by cells in a timely manner provide the coordinates that delineate their individual fate. Extrinsic factors influence cells as groups through the process of differentiation. The integration of intrinsic and extrinsic cues is the decisive denominator providing the coordinates of cell fate and differentiation. By understanding the interplay between these factors we will hopefully dissect the processes leading to cell fate assignment. Research data from the last two decades suggest that there are six main signaling transduction pathways in the cell (Martinez Arias and Stewart, 2002): Wnt (Wingless/Int-1), Hedgehog (Hh), Notch, receptor tyrosine kinase (RTK), steroid hormone receptor, and bone morphogenic proteins (BMP). They are thought to act in parallel, to ultimately enhance specific genes that result in cell type-specific combinations of transcription factors responsible for cellular behavior (Martinez Arias and Stewart, 2002; Barolo and Posakony, 2002), representing the basic machinery for the determination of embryonic cell fate determination. Examples include the photoreceptor cellular fate determination in the *Drosophila* model (Silver and Rebay, 2005; Voas and Rebay, 2004) and in the eight-cell embryo of *Caenorhabditis elegans* during the formation of blastomeres (Rose and Kemphues, 1998; Newman-Smith and Rothman, 1998; Platzer and Meinzer, 2004). The combined activity of transcription factors in these examples is defined via specific temporospatial

coordinates. There is increasing evidence suggesting that there are subtler interactions between components of the signaling transduction pathways controlling cell fate during development and we will now move on to explore these entities in the context of the lung, firstly by describing their most important interactions, both during development and tissue maintenance and during carcinogenesis. We will also consider the microenvironment, a crucial factor to stem cells, providing the necessary signs for their correct regulation and function, and we will investigate how anomalies in this may contribute to the process of malignant conversion.

2.1 The Wnt Signaling Pathway in Lung Cancer

The Wnt pathway was named after the *wingless* gene, the *Drosophila* homologous gene of the first mammalian Wnt gene characterized, *int-1* (Rijsewijk et al., 1987). Wnt signaling occurs upon the binding of secreted Wnt ligands, triggering changes in gene expression, cell behavior, adhesion, and polarity. In mammals, Wnt proteins comprise a family of 19 highly conserved cysteine-rich signaling glycoproteins. In the human adult lung Wnt2, Wnt5a, and Wnt11 are expressed in the mesenchyme and Wnt7 is expressed in lung epithelium (Wang et al., 2005; Lako et al., 1998; Li et al., 2002; Shu et al., 2002). So far, Wnt transduction has been described in at least three pathways (Widelitz, 2005). The most characterized Wnt pathway is the canonical signaling cascade, in which Wnt ligands bind to two distinct families of cell surface receptors, the Frizzled (Fz) receptor family and the LDL receptor-related protein (LRP) family, and activate target genes through the stabilization of beta-catenin in the nucleus (Akiyama, 2000) (Fig. 1). In the non-canonical Wnt/Ca2+ pathway, Wnt proteins signal through the activation of calmodulin kinase II and protein kinase C, leading to an increase in intracellular Ca2+. Wnt can also signal non-canonically through Jun N-terminal kinase (JNK); this is known as the planar cell polarity pathway, controlling cytoskeletal rearrangements (Veeman et al., 2003).

2.1.1 The Canonical Wnt Pathway

The canonical Wnt pathway becomes active when Wnt ligands bind to respective Frizzled (Fz) receptors and low-density lipoprotein receptor-related proteins-5/6 (LRP5/6) co-receptors. Subsequently activation of the cytoplasmic phosphoprotein, Disheveled (Dvl), causes inhibition of a cytoplasmic complex made of glycogen synthase kinase 3 (GSK-3), Axin, and adenomatous polyposis coli (APC), to then repress the phosphorylation of beta-catenin by GSK-3 (Nusse, 2005). Stabilization and release of hypophosphorylated beta-catenin leads to cytoplasmic accumulation and translocation with the help of BCL9, to the nucleus (Kramps et al., 2002; Krieghoff et al., 2006; Sampietro et al., 2006). In

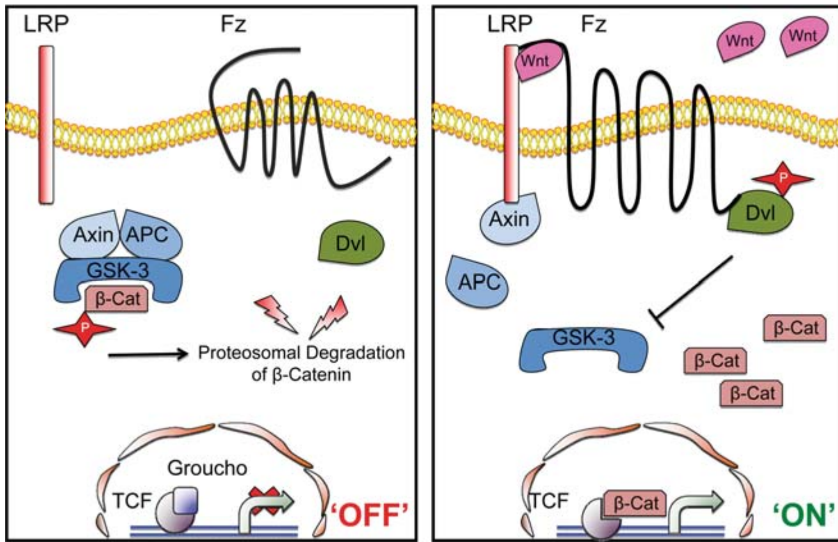

Fig. 1 The canonical Wnt transduction pathway.
Proteosomal degradation of beta-catenin via its phosphorylation occurs in the absence of Wnt ligands. Downstream Wnt target genes are maintained repressed ('*OFF*'). Degradation of active beta-catenin is reduced upon binding of Wnts. Accumulation and translocation of beta-catenin into the nucleus leads to binding to T-cell factors and activation of target genes ('*ON*'). *APC* adenomatous polyposis coli, *Dvl* Disheveled, *GSK* glycogen synthase kinase, *TCF* T-cell factor

the nucleus it binds to DNA-bound T-cell factor (TCF)/lymphoid enhancer protein (LEF) family members to initiate the transcription of downstream target genes. In the absence of the Wnt signal, the TCF/DNA-binding proteins form a complex with Groucho and repress Wnt target genes (Cavallo et al., 1998), (Nusse, 2005; Logan and Nusse, 2004). Groucho can interact with histone deacetylases making the DNA refractory to transcriptional activation (Chen et al., 1999a). Upon nuclear entry of beta-catenin into the nucleus, it competes with Groucho for binding to TCF/LEF, recruits Pygopus, and converts the TCF repressor complex into a transcriptional activator complex (Fig. 1). Target genes include c-Myc, cyclin D1, MMP7, and WISP and a comprehensive list of other Wnt target genes may be found on the Internet at http://www.stanford.edu/~rnusse/wntwindow.html.

The Wnt pathway plays a critical role in lung carcinogenesis. Expression of the Wnt inhibitor Dickkopf-1 (Dkk-1) has been shown to occur in the distal epithelium of pulmonary airways; knockout experiments have shown that Dkk-1 inhibits branching morphogenesis (De Langhe et al., 2005) and aberrant Wnt pathway has been shown to have a role in non-small cell lung cancer

Table 1 Examples of Wnt transduction in human cancers

Tumor	Authors	Notes
Adenocarcinoma of the colon	Suzuki et al. (2004)	Restoration of SFRP in colorectal cancer cells limits WNT signaling
Adenocarcinoma of the lung	You et al. (2004)	Inhibition of Wnt2-mediated signaling induces apoptosis through inactivation of Survivin
Lymphoblastic leukemia	Chung et al. (2002)	Overexpression of dominant negative beta-catenin or dominant TCF inhibits proliferation
Multiple myeloma	Derksen et al. (2004)	Stimulation of growth by Wnt ligands. Dominant negative TCF-4 inhibits growth
Oral squamous cell carcinoma	Sogabe et al. (2008)	Ectopic expression of SFRPs inhibits cancer cell proliferation in vitro
Osteosarcoma	Hoang et al. (2004a,b)	Expression of Dkk-3 and LRP5 inhibits cancer cell growth in vitro
Pleural mesothelioma	Lee et al. (2004)	Restoration by transfection of the SFRP gene construct into cell lines lacking SFRP expression results in apoptosis and growth suppression

(NSCLC) (Table 1). Indeed, Wnt2 is overexpressed in NSCLC and inhibition of Wnt2-mediated signaling by siRNA or a monoclonal antibody leads to apoptosis in NSCLC cell lines (You et al., 2004). Wnt inhibitory factor (WIF1), a secreted antagonist of Wnt, can inhibit growth both in vitro and in vivo in lung adenocarcinoma cell lines (Kim et al., 2007). Hypermethylation of the WIF1 promoter is a common mechanism of anomalous silencing of WIF1 which leads to activation of Wnt signaling in lung cancer (He et al., 2005). Abnormally high expression of Disheveled 3 (Dvl-3), a positive regulator of Wnt, has been shown to occur in about 75% of freshly microdissected NSCLC tumor specimens (Uematsu et al., 2003).

2.1.2 The Non-canonical Wnt Pathway

In the Wnt/Ca2+ pathway, Wnt proteins signal through the activation of calmodulin kinase II and protein kinase C, leading to an increase of intracellular Ca2+. Cell polarity can be influenced through Wnt via the Jun N-terminal kinase (JNK) signaling cascade (Veeman et al., 2003). Wnt has also been described to atypically signal via the receptor tyrosine kinase (RTK) pathway (Oishi et al., 2003) and through a newly discovered cascade involving the cyclic adenosine monophosphate (cAMP) pathway activating protein kinase A downstream of specific Wnts ultimately affecting myogenesis; the phosphorylation and activation of the cAMP response element-binding (CREB) (Chen et al., 2005) is a protagonist in this signaling pathway.

2.1.3 Wnt Ligands

Wnt proteins are secreted highly conserved, cysteine-rich, glycoproteins of approximately 40 kDa in size. To date, 19 Wnt proteins have been identified in humans in a variety of cells (Miller, 2002). Cysteine palmitoylation is required for the function of Wnt and cells that secrete Wnt proteins require Porcupine (Porc), a homologous to some endoplasmic reticulum acyltransferases, suggesting that Porc could be responsible for cysteine palmitoylation of Wnts (Hofmann, 2000; Willert et al., 2003; Zhai et al., 2004). Extracellular heparan sulfate proteoglycans (HSPGs) also seem to be involved in the transportation and stabilization of Wnt proteins (Banziger et al., 2006; Bartscherer et al., 2006; Lin, 2004). Clearly, a lot remains unknown in this field of Wnt signaling.

2.1.4 Wnt Receptors and Antagonists

We have seen how Wnt signaling is initiated after Wnt proteins bind to members of two distinct families of cell surface receptors, the Frizzled (Fz) protein family and the LDL receptor-related protein (LRP). Wnt ligands bind to Fz proteins through an extracellular N-terminal cysteine-rich domain (CRD). There are ten Fz proteins that have been characterized so far. Their molecular structure is similar to that of the seven-transmembrane G protein-coupled receptors perhaps indicating that Fz proteins may use heterotrimeric G proteins to transduce their signal (Bhanot et al., 1996; Liu et al., 2001). Furthermore, cell surface receptors LRP5 or LRP6 (single-pass transmembrane molecules of the LRP family of receptors) are needed together with Fz for Wnt signaling. The hypothesis of a trimeric complex between Wnt molecules, Fz, and LRP5/6 remains under scrutiny (Tamai et al., 2000). Derailed and Ror2 are two tyrosine kinase receptors that bind to Wnts. Derailed couples to Wnt via an extracellular WIF (Wnt inhibitory factor) domain, and Ror2 does it through a Wnt CRD motif (Lu et al., 2004; Mikels and Nusse, 2006). Secreted Frizzled-related proteins (SFRPs) and the Wnt inhibitory factor-1 (WIF1) are good examples of inhibitory proteins that can sequester Wnt ligands from their receptors. There are five SFRP members in humans each containing a CRD domain. The complex biology of SFRPs is exemplified through their function as agonists and/or antagonists of Wnt (Hsieh et al., 1999; Jones and Jomary, 2002; Uren et al., 2000). WIF1 contains a unique, evolutionarily conserved WIF domain and five-epidermal growth factor (EGF)-like repeats that show no homology to SFRPs. The Dickkopf (Dkk) family of extracellular Wnt inhibitors represents a third class of Wnt inhibitors that antagonize Wnt via the inactivation of LRP5/6 (Fedi et al., 1999).

2.2 The Hedgehog Signaling Pathway in Lung Cancer

Hh signaling transduction exerts a role in embryonic patterning as well as stem cell proliferation, growth, and tissue homeostasis in the *Drosophila* and in humans as well (Varjosalo and Taipale, 2007). It has been shown that aberrant

Hh signaling is responsible for many developmental malformations (Ingham, 1998; Goodrich and Scott, 1998; McMahon, 2000; Lee et al., 1992; Kalderon, 2000). Hh was identified as a secreted signaling protein necessary for specification of positional identity in the *Drosophila* embryonic segment and in patterning of imaginal disc-derived adult structures such as the appendages, the eye, and the abdominal cuticle (Ingham, 1998; Goodrich and Scott, 1998; McMahon, 2000; Lee et al., 1992; Kalderon, 2000). The Hh signaling transduction cascade commences upon the intramolecular cleavage and lipid modification of Hh. The Hh protein precursor carboxyl-terminal portion catalyzes this reaction. The resulting amino-terminal peptide, which is esterified on its C terminus to a cholesterol molecule (HhNp), is responsible for most actions of the Hh protein. In mammals ShhNp proteins undergo further palmitoylation at their N termini. This additional process, which is dependent on prior addition of a cholesterol group, augments the activity of the protein in certain cellular contexts (Porter et al., 1996; Pepinsky et al., 1998). Intracellular Hh signaling, in contrast to other pathways, occurs by chronological repressive interactions. Two transmembrane proteins, the tumor-suppressor Patched (Ptch) and the proto-oncogene Smoothened (Smo), regulate Hh initiation and transduction (Ingham, 1998; Goodrich and Scott, 1998; Kalderon, 2000). Ptch is a 12-span transmembrane protein and Smo is a member of the seven-transmembrane family of receptors resembling the Fz receptor family in the Wnt pathway. During the 'off' state of the Hh pathway Smo is suppressed by Ptch. Upon stimulation by Hh, the inhibition of Smo is released, and Hh signaling commences. The heteromeric receptor model for the Hh pathways proposes interaction between Hh and Ptch and between Ptch and Smo; in this model Hh binds to Ptch within the Ptch/Smo complex, releasing the activity of Smo without the disintegration of Ptch and Smo. Dissimilar in vivo localization of Ptch and Smo, despite physiological relationships between Hh and Ptch, suggests that alternative models to explain this pathway must be explored (Kalderon, 2000; Denef et al., 2000; Stone et al., 1996). The mechanisms through which activation of Smo relates to some of the cytoplasmic components of the Hh pathway, including the serine/threonine protein kinase Fused (Fu), Supressor of Fused (Su(fu)), the kinesin-like protein Costal-2 (Cos2), and the transcription factor Cubitus interruptus (Ci; Gli in mammals), remain to be understood (Ingham, 1998; Goodrich and Scott, 1998; Denef et al., 2000; Taipale et al., 2000; Aza-Blanc et al., 1997). In *Drosophila* these molecules aggregate upon their interaction to form a microtubule-anchored cytoplasmic complex through the action of Cos2. In the absence of Hh, protein kinase A phosphorylates Ci (Ci155), which is then cleaved into an N-terminal transcriptional repressor (Ci75) (Aza-Blanc et al., 1997). Hh signaling results in disintegration of the cytoplasmic complex from the microtubules, followed by nuclear translocation of the full-length Ci transcriptional activator, which leads to transcriptional activation of Hh target genes (Robbins et al., 1997; Chen et al., 1999b), including *Ptch*. This in turn results in an inhibitory feedback process (Freeman, 2000). In mammals there are three *Hh* genes: *Sonic, Indian,* and *Desert hedgehog* (*Shh, Ihh,* and *Dhh)*. Several but not all homologues of the *Drosophila* Hh cytoplasmic proteins have

been identified, many of which have multiple isoforms. The inhibitory transcriptional function of Ci in mammals seems to occur through Gli-3, while Gli-1 and Gli-2 behave as activators of the pathway. However, a lot remains unknown in the mammalian Hh pathway and its complexities.

Epithelial–mesenchymal tissue interactions control the branching of embryological murine pulmonary buds, when expression of Shh is at its highest level. These levels decrease becoming very low around birth, though remaining detectable throughout the epithelium postnatally (van Tuyl and Post, 2000; Bellusci et al., 1997). Shh-null mice exhibit hypoblastic lung buds without airway branching with serious consequences to viability. In contrast, transgenic overexpression of Shh leads to the absence of functional alveoli and hyperproliferation of epithelial and mesenchymal pulmonary cells (Bellusci et al., 1997; Litingtung et al., 1998). In adult lungs, only a small number of basal bronchial epithelial cells show constitutive hedgehog signaling. Mice exposed to naphthalene airway damage demonstrate an expansion of the intraepithelial cell population with active Hh during airway regeneration. Interestingly, the expansion of these regenerative cells precedes the increase of a rare pulmonary neuroendocrine population of cells, which is considered to be a potential stem epithelial progenitor (Litingtung et al., 1998; Reynolds et al., 2000a). The growth of SCLC cell lines, which maintains components of the Hedgehog pathway and with features of primitive neuroendocrine differentiation, was inhibited by cyclopamine, a naturally occurring steroidal alkaloid inhibitor of Smoothened and therefore the Hedgehog pathway. These results, however, were not observed in NSCLC cell lines (Cooper et al., 1998; Watkins et al., 2003) indicating that progenitor respiratory epithelial cells may follow a neuroendocrine route in response to Hedgehog signaling elicited by neighboring cells. In contrast, SCLC could be the result of unchanged and unregulated Hedgehog signaling. Similar to the case of Wnt and Notch, the robust identification of putative CSCs in the lung and how this pathway may influence them is essential.

2.3 The Notch Signaling Pathway in Lung Cancer

The Notch protein belongs to a family of single-transmembrane-domain receptors that have an extracellular domain consisting of EGF-like repeats and an intracellular domain with seven Ankyrin (ANK) repeats (Ehebauer et al., 2005; Nam et al., 2003, 2006; Zweifel et al., 2003). The Notch intracellular domain (NICD) acts as a membrane-bound transcription factor. Binding of Notch to its ligands Serrate and Delta releases NICD (Kopan, 2002). Free NICD translocates into the nucleus, to interact with CSL (CBF in vertebrates, Suppressor of Hairless [Su(H)] in *Drosophila*, and LAG-1 in *C. elegans*), to initiate the transcription of target genes. Endocytic trafficking and/or the localization of Notch to a specific endocytic compartment is necessary for the cleavage of NICD

(Bray, 2006; Ehebauer et al., 2006; Le Borgne, 2006; Jaekel and Klein, 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005).

There is increasing evidence on the specific roles of the individual components of this pathway in both organogenesis and tissue maintenance throughout the tracheobronchopulmonary tree (Bettenhausen et al., 1995; Juopperi et al., 2007; Post et al., 2000). Interestingly, newer studies are involving Notch in the maintenance of lung CSCs; NSCLC cell lines were found to express Notch-1 and Notch-3 as well as their downstream effector Hes-1. Interestingly, these proteins were not found in small cell lung cancer (SCLC) cell lines (Chen et al., 1997; Collins et al., 2004; Dang et al., 2000). Neuroendocrine differentiation in SCLC depends, in part, on the action of the basic-helix-loop-helix (bHLH) transcription factor human achaete-scute homologue-1 (hASH1). In contrast to Notch-1 and Notch-3, hASH1 expression, which is normally inhibited through Notch, is elevated in neuroendocrine SCLC but seldom in NSCLC (Ball et al., 1993). These studies suggest that Notch (essential in Clara cell and neuroendocrine cell development) is aberrantly regulated in NSCLC and SCLC hinting toward further potential roles in stem cell fate.

2.4 The Importance of Communication Between Developmental Pathways

We have highlighted some of the crucial developmental cell signaling pathways and some of the evidence demonstrating their involvement in normal lung organogenesis with the aim to then explore how they play a role in tissue homeostasis and how this is exerted through their action on stem and progenitor pulmonary cells within specific niches or microenvironments. Before we do that, we must emphasize the importance of crosstalk among these pathways and the complex relationships between their components. These often occur in synchronous enhancing or opposing manners, ultimately orchestrating the function of rare pluripotent cells in the bronchopulmonary tree maintaining adequate lung function. Understanding these mechanisms of interaction and their real functional significance is as important as understanding the individual pathways on their own.

Wnt and Hh signalings are fundamental for the development of diverse epithelial tissues such as the teeth, the gastrointestinal tract, and hair follicles (Logan and Nusse, 2004; Hooper and Scott, 2005; Gregorieff and Clevers, 2005). Ectopic activation of these pathways can lead to malignancy (Tables 1 and 2), and inhibition of their function is the culprit of many developmental abnormalities (Taipale and Beachy, 2001; McMahon et al., 2003). Some proof regarding the relationship between Wnt and Hh has been reported during embryonic organogenesis and both pathways have shown protagonism in epithelial–mesenchymal interactions; the precise mechanisms dictating these mutual synergistic actions continue to be fairly mysterious (Noramly et al.,

Table 2 Examples of Hh transduction in human cancers

Tumor	Authors	Notes
Adenocarcinoma of the biliary tree	Berman et al. (2003)	Cyclopamine and Hh blocking antibody inhibit cancer cell line proliferation
Adenocarcinoma of the esophagus	Berman et al. (2003)	Cyclopamine and Hh blocking antibody inhibit cancer cell line proliferation
Adenocarcinoma of the stomach	Berman et al. (2002)	Cyclopamine and Hh blocking antibody inhibit cancer cell line proliferation
Medulloblastoma of the brain	Berman et al. (2002)	Inactivation of Ptch leads to cancer growth and cyclopamine inhibits cancer allograft and cell line growth
Oral squamous cell carcinoma	Nishimaki et al. (2004)	Cyclopamine inhibits cancer cell line proliferation
Small cell lung cancer	Watkins et al. (2003)	Cyclopamine and Hh blocking antibody inhibit cancer cell line proliferation and xenograft growth

1999; Bitgood and McMahon, 1995; Reddy et al., 2001; Heemskerk and DiNardo, 1994; Silva-Vargas et al., 2005; Iwatsuki et al., 2007). In cancer the link between Wnt and Hh is also well established (Tables 1 and 2), as shown by van den Brink et al.; their experiments elegantly demonstrated that Ihh is a negative regulating factor of Wnt during epithelial differentiation in the colon (van den Brink et al., 2004). Proliferation of colorectal cancer cells through Wnt suppression occurred through Gli-1 (Akiyoshi et al., 2006). Another group found that Gli-1 was able to inhibit Wnt through SFRP-1, an antagonist of Wnt and a transcriptional target of Hh (Akiyoshi et al., 2006). As the intricacies of Hh are understood newer cues on how this developmental pathway interacts with Wnt are brought to light; in turn this knowledge will aid understanding how the function of pluripotent and progenitor pulmonary cells is regulated.

Evidence regarding the interplay between Wingless and Notch was first discovered in the context of wing patterning and development in *Drosophila* (Couso and Martinez Arias, 1994; Hing et al., 1994). Initially they synergize in the early formation of the wing primordium. Later, Notch enhances *wingless* expression at the future wing margin (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996). The detailed genetic experiments in organisms such as *Drosophila* that led to the discovery of some crucial mechanisms of interaction between Wnt and Notch are not possible in vertebrates due to technical and logistic reasons. However, despite the limitations, there is evidence that in vertebrates also, mutual interactions between *Notch* and *Wingless* do occur during somitogenesis and muscle formation (Aulehla et al., 2003; Aulehla and Herrmann, 2004), during the formation of skin precursors (Estrach et al., 2006), and during the patterning of rhombomeres (Cheng et al., 2004).

There are developmental circumstances in which Wnt signaling is linked to high Notch signaling and vice versa, showing clear antagonism. In other circumstances, such as during somitogenesis, where the central pattern generator of development is the Notch-driven spatiotemporal cycles of gene expression, it has been possible to identify the regulatory motif in which Delta expression is under the control of Wnt signaling transduction (Aulehla et al., 2003; Aulehla and Herrmann, 2004; Galceran et al., 2004; Pourquie, 2003). When the results of these studies are considered together, one may conclude that the Wnt and Notch signaling pathways have an interactive relationship during development.

3 The Stem Cell Niche

Stem cells are acquiring significant importance as valuable research tools and as a potential resource for new cell substitution therapies for benign and malignant illnesses. Research has shown that the specific microenvironment in which stem cells dwell is essential for their adequate function. As such, we need to explore the concept of stem cell microenvironments and how they provide a platform for developmental pathways and stem cells to interact, affecting their ultimate cellular fate. A revolution in the field of stem cells occurred in 1998 when Thomson et al. isolated human embryonic stem cells (hESCs). Many speculative reports on somatic stem cell plasticity promoted the revolution ironically paving the basis for regenerative medicine. In biological terms, the concept of a microenvironment or niche in a solely architectural or geographical sense is insufficient; a biological niche must also exert a functional sustaining role as well as an anatomical dimension that facilitates resident stem cells to self-renew. Studies have demonstrated that somatic stem cells have limited function outside their microenvironment. Good examples of this are hematopoietic stem cells, which have the power to regenerate successfully the entire blood and immune system. However, these cells have little function outside specific cellular niches where specific signals push them to follow physiologically appropriate fates. Comprehending how the niche modulates stem cells will hopefully provide the appropriate tools to therapeutically manipulate stem cells allowing new ways to treat malformative, degenerative, and cancerous pathologies. We will now bring to light some of the most relevant aspects of stem cell niches and the evidence behind their importance.

3.1 Niche Infrastructure: The Extracellular Matrix

Studies in *Drosophila* and *C. elegans* have shown that germ stem cells in these organisms localize at the distal end of a tapered structure. These stem cells communicate with somatic cells that help them maintain their 'stemness'

within the structure (Crittenden et al., 2002; Xie and Spradling, 2000; Kiger et al., 2000). These experiments suggest that the niche contains heterologous cell populations. Two studies in *Drosophila* showing that posterior mid-gut cell populations yield daughter enterocytes and enteroendocrine raised the controversy on whether there is need for other cell types for the functionality of the niche. In these studies stem cells did not necessitate direct contact with heterologous cells. Armadillo (a beta-catenin paralog) localized specifically at the interface between stem cells (on the basement membrane separating them from adjacent muscle cells) and their resulting enteroblasts. This suggests that it is perhaps the basement membrane that provides a specific microenvironment within the intestine. This microenvironment or niche is composed of extracellular matrix and other non-cellular elements that in unison control the cells within that niche without the necessity of heterologous cells. In mammalian skin, β-1 integrins have been found to be differentially expressed on primitive cells and to engage in constrained localization of a stem cell population via presumed crosstalk with matrix glycoprotein ligands (Jones and Watt, 1993; Jensen et al., 1999). In the nervous system, lack of Tenascin C changes neural stem cell number and function in the subventricular zone (Garcion et al., 2004). In the blood system, deletion of Tenascin C affects primitive cell populations, suggesting a role in the regulation of hematopoietic stem (HS)-cell niches (Ohta et al., 1998). Osteopontin (OPN), a matrix protein that is also involved in cell-mediated immunity and metastasis, has recently been shown to play an important function in the regulation of hematopoietic stem cell niches. OPN production fluctuates with osteoblast activation, and animals deficient in OPN show an increased number of HS cells. OPN was shown to limit the number of stem cells under homeostatic conditions or with stimulation, behaving as some sort of constraint regulating hematopoietic stem cells. In mammals examples of niches include the identification of osteoblasts in the bone marrow compartment, and the endothelium in the brain (Palmer et al., 2000; Calvi et al., 2003; Zhang et al., 2003; Kiel et al., 2005). These studies show that extracellular matrix components confer localizing niches that may provide stimulatory and or inhibitory effects on the stem cells.

3.2 Paracrine Signals Within the Niche

The signals governing the physical organization of the niche are an important component of the machinery of the niche. Experiments in mice have shown that ephrins and their RTK receptors are mediators of structural boundaries and they are involved in the organization of epithelial cells in the intestines. Anomalous expression of these molecules was found to lead to abnormal organization of the crypt and the intestinal villus, affecting the stem cells contained within. Wnt proteins as well as their antagonists regulate in a paracrine manner, to

impair differentiation and to promote proliferation of the cells composing the organizing structure of the niche. As such, studying those Wnt molecular events is important to understand how their aberrant regulation leads to cancer initiation and progression (Batlle et al., 2002), and this is certainly work in progress. In conclusion, so far, matrix glycoproteins and the three-dimensional spaces that they form are the ultrastructure of the stem cell niche. Secreted proteins (by Wnt and other developmental pathways) offer a paracrine level of control on the stem cells and the ultrastructure that holds them, playing a crucial role in their cellular behavior and fate.

3.3 The Concept of Stem Cells and Cancer Stem Cells

Tumor tissue architecture shows many features of normal structures, with a cellular hierarchy that regulates the balance between cell renewal and cell differentiation. Interactions between cancer and stromal cells rely on deregulated physiological feedback mechanisms that in normal circumstances are responsible for tissue maintenance (Szabowski et al., 2000; Donjacour and Cunha, 1991; Sternlicht et al., 1999; Muller et al., 2001). Normal stem cells

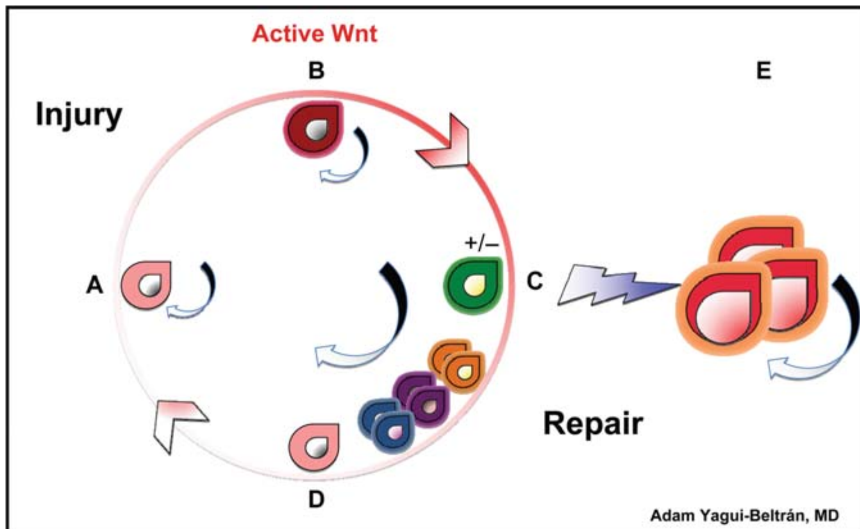

Fig. 2 Tissue homeostasis and carcinogenesis through stem cell cycling.
Quiescent stem cell (SC) with inactive Wnt: **A**. Upon tissue trauma, Wnt transduction leads to activation of homeostatic SCs: **B**. These cells produce more pluripotent SCs as well as progenitor cells with limited proliferative power: **C** that produce specialized differentiated cells (shown in *orange*, *purple*, and *blue*) in order to regenerate the tissue. Upon repair, SCs cycle into a quiescent state: **D** and **A**. Accumulation of oncogenic events may 'lock' activated SCs in a permanent Wnt-driven state leading to cancer stem cells: **E** (Adapted from Beachy et al., 2004)

must have three characteristics: the ability to self-renew to allow maintenance of a population of undifferentiated stem cell pool throughout life; a strict regulation of stem cell numbers; and the capacity to differentiate in order to clonally repopulate functional cells within a tissue (Al-Hajj et al., 2004). Stem cells differ in their intrinsic ability to self-renew and differentiate (Bixby et al., 2002). The concept of 'cancer stem cell' describes a cancer cell that has the power to self-renew resulting in another cancerous stem cell as well as a cell that will give rise to the phenotypically diverse cancer cell populations (Wicha et al., 2006; Sell, 2004; Houghton et al., 2007) (Figs. 2 and 3). These latter cells are thought to represent the bulk-tumor proliferative cell pool that responds to chemoradiotherapy, leaving the cancer stem cell population unaffected. Eventually this results in tumor repopulation and disease recurrence (Houghton et al., 2007). Cancer stem cells were initially discovered in hematological cancers. After transplantation, only a small subpopulation of these cells was able to extensively proliferate and repopulate (Park et al., 1971; Bruce and Van Der Gaag, 1963; Wodinsky and Kensler, 1966; Bergsagel and Valeriote, 1968). In the cancer stem cell model, the disruption of genes responsible for the regulation of self-renewal is predictably important. There is considerable evidence demonstrating that only a subset of cells in a particular cancer displays characteristics of self-renewal. It is proposed that the microenvironment or the niche, as explained

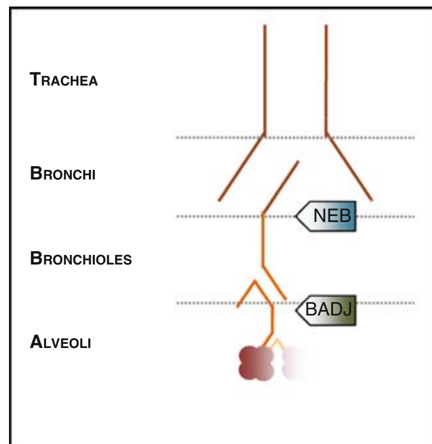

Fig. 3 Stem cell niches in the tracheobronchopulmonary tree
Diagram of the human airway and some of the lung niches along its proximal-to-distal tracheopulmonary distribution. These distinct microenvironments are thought to harvest a diverse group of pulmonary stem cells that may be responsible for the heterogeneous types of lung carcinomas. Squamous cell carcinomas arise in the upper airways in a transitional manner from the submucosal glands. Neuroendocrine cells are likely to be responsible for small cell carcinomas. Stem cells localizing distally in the airway may be the culprits for bronchoalveolar carcinomas as well as adenocarcinomas of the lung. *NEB* neuroepithelial body, *BADJ* bronchioalveolar duct junction

above, provides the necessary signals needed for the stem cells to continue escaping the constraints that normally restrict their capacity to self-renew and allows them, upon exit from their niche, to undergo differentiation.

3.3.1 Pulmonary Stem Cell Niches

There is accumulating and robust scientific proof supporting that lung stem cells may be some of the culprits in lung carcinogenesis. Many experiments have shown that certain malignant lung tumors contain specific subpopulations of cancerous stem cells that are resistant to chemotoxic agents (Al-Hajj et al., 2003; Singh et al., 2004; Kondo et al., 2004; Patrawala et al., 2005; Hirschmann-Jax et al., 2005; Galli et al., 2004; Hemmati et al., 2003), which is intrinsically exciting because the identification and targeting of those cancerous stem cells opens up the possibility of novel therapeutic agents against lung cancer, and cumulative permanent mutations in stem cells and the pathways regulating them and their niche are needed to expand their populations and eventually lead to cancer (Hochedlinger et al., 2005). However, a lot remains unknown on whether normal and cancer stem cells, with distinctive cellular differences and yet with the same potential to differentiate and proliferate, share a common cellular origin. Transgenic mouse models are being used to explore these questions, to evaluate the single oncogenic mutations that contribute to the process; and to figure out how they deregulate physiological tissue maintenance exerted through specific signaling cascades; and to then understand how they may induce and perpetuate tumor phenotypes (Hochedlinger et al., 2005; Fisher et al., 2001; Lo Celso et al., 2004). Organs with intrinsically highly dividing cells, such as the blood, the gastrointestinal tract, and the skin, are more likely to accumulate carcinogenic mutations with time. On the other hand, organs with more committed progenitor cells and shorter lifespan have less time to accumulate permanent mutations required for the malignant transformation of physiological homeostatic stem cells. It is not surprising that in mutagenic animal models in which committed daughter cells are targeted, the identification of cancer stem cells has been unsuccessful (Barrandon et al., 1989; Pelengaris et al., 1999; Owens and Watt, 2003). It is well known that lung cancer is a group of regionally and phenotypically distinctive malignant diseases and although theoretically only some oncogenic mutations may occur, what actually happens to potential stem cells with slower cell turnover and longer tissue transit time remains unclear (Passegue et al., 2003). In transgenic mouse models, the diverse subclasses of pulmonary cancers follow a proximal-to-distal tracheopulmonary distribution, and these studies are helping elucidate the link between pulmonary microenvironments and the cells that they contain. Deciphering how unique tumorigenic properties are associated with discreet cellular mutations is helping in the understanding of how individual stem cells are capable of generating phenotypically similar lung cancers (Meuwissen and Berns, 2005; Franklin et al., 1997). Squamous cell carcinomas (SCC) of the trachea are centrally located lesions; small cell lung carcinomas (SCLC) and adenocarcinoma/bronchoalveolar neoplasias

grow in a more distal fashion. The tumor regionality observed in the mouse may suggest that there are a defined number of distinct pulmonary niches and cells capable of initiating, promoting, and sustaining the carcinogenic cascade. There is evidence that humans also display pulmonary tumor regionality (Fig. 3), although thus far, the exact location of potential lung cancer stem cells is still unknown. A classical lung cancer mouse model is the mutant pan-pulmonary K-ras model. In these animals only non-cancerous adenomatous hyperplastic lesions exclusively localized to the bronchoalveolar region despite the fact that identical mutations had been universally induced through the tracheobroncho-pulmonary apparatus (Fisher et al., 2001; Meuwissen et al., 2001; Johnson et al., 2001; Jackson et al., 2001; Guerra et al., 2003). Other mouse models have demonstrated that bronchiolar stem cells, within neuroepithelial body microenvironments (NEB), have been linked to SCLC (Watkins et al., 2003; Collins et al., 2004; Ball et al., 1993; Giangreco et al., 2004; Williams et al., 1994; Wikenheiser-Brokamp, 2004; Meuwissen et al., 2003; Minna et al., 2003; Reynolds et al., 2000b; Linnoila et al., 2000, 2005; Van Lommel et al., 1999; Miller et al., 2001; Giangreco et al., 2007) and central bronchiolar adenocarcinoma and bronchoalveolar cell carcinomas have been shown to arise within distal pulmonary niches, from the bronchoalveolar duct junction (BADJ) harvesting bronchioalveolar stem cells (BASCs). These BASCs have been identified, expanded, and characterized in mice (Kim et al., 2005). Although, in general, human lung cancers either exhibit airway or alveolar differentiation some types of cancers exhibit both airway and alveolar differentiation, which is similar to what is found in most mouse models (Kim et al., 2005). The working hypothesis for potential human BASCs is that either Clara or alveolar type 2 (AT2) cells give origin to the cellular precursors of adenocarcinomas (Fisher et al., 2001; Kim et al., 2005). There is increasing evidence from the murine adenocarcinoma models that in humans, precursor cells for adeno and bronchoalveolar carcinoma also localize to the BADJ zone (Meuwissen and Berns, 2005; Meuwissen et al., 2001; Jackson et al., 2001; Politi et al., 2006; Ji et al., 2006; Bottinger et al., 1997; Wikenheiser et al., 1992; DeMayo et al., 1991). These examples show how different histological types of lung cancer are linked to specific pulmonary microenvironments with unique stem cells and will hopefully lead to the identification of such entities in the human lung, with obvious potentially beneficial new therapeutic targets (Fig. 3).

3.3.2 Identification of Pulmonary Cancer Stem Cells

The presence of a clonogenic population of cells in human lung cancer was described almost three decades ago. Clinical specimens from SCLC and adenocarcinoma patients were found to contain a small subpopulation of cells ($<1.5\%$) that possessed the ability to form colonies when grown on agar. Upon their intracranial injection into athymic nude mice, they yielded cancers with features identical to those of the original specimens. This supports the notion of cancer stem cell (CSC) populations within some lung cancers (Carney et al.,

1982). Elevated expression of ABC transporters is associated with an increased resistance to chemotoxic agents compared to non-side-population (SP) cells (Goodell et al., 1996; Gutova et al., 2007). SP cells isolated by the efflux of Hoeschst 33342 by ABC transporters with stem cell characteristics have been characterized in NSCLC and in clinical specimens of lung cancer cell lines. Further evidence supporting that CSCs have features of immortality and quiescence is demonstrated by the fact that certain SP cells contain high levels of telomerase mRNA and decreased levels of MCM7, a proliferation marker (Wicha et al., 2006; Ho et al., 2007). Purified SP cells were cultured in vitro and showed a greater invasion potential. They produced not only more SP subpopulations but also non-SP subsets, repopulating the original presorted cell line. Furthermore in vivo inoculation of these SP cells into mice proved that smaller number of cells (inoculums) were required to generate malignant xenografts in non-obese diabetic-severe combined immunodeficiency mice, suggesting that these cell populations had increased tumorigenicity compared to non-SP cells (Ho et al., 2007).

We will now explore the most important cell surface markers in pulmonary stem cells and highlight their contribution to the advancement of our knowledge in this field.

Cell Surface Markers

The robust identification and targeting of human pulmonary CSCs will lead to novel modalities for lung cancer diagnosis and treatment. Enormous financial efforts are being made toward finding those small cellular populations responsible for resistance to traditional cytotoxic therapies. The identification of stout CSCs markers that are expressed extracellularly and independent of functional stem cell assays is therefore important to dissect the functional mechanisms leading to the transformation of normal stem cells into CSCs (Lowry and Richter, 2007).

CD117 (c-Kit)

Expression of CD117 and its ligand, a stem cell factor in pulmonary neuroendocrine tumors, was identified in the 1990s (Hibi et al., 1991). CD117 is related to poor prognosis in early stage lung adenocarcinoma and squamous cancer patients, even though in advanced disease its expression is only found in about a third of patients (Pelosi et al., 2004). Interestingly, phase II clinical trials failed to demonstrate the clinical efficacy of the Abl/c-Kit/PDGF inhibitor (Imatinib) in lung cancer, and no mutations of CD117 were found in these tumors, in contrast to GI stromal tumors (GIST). This inefficacy was also true for lung cancer patients selected for expression of c-Kit, suggesting that CD117 is unlikely to mark an early progenitor in lung cancer (Altundag et al., 2005; Dy et al., 2005; Gross et al., 2006).

CD44

CD44 is a ubiquitously expressed multispan transmembrane cell surface adhesion glycoprotein. It has functions in cell–matrix and cell–cell interactions and it has been linked to poor prognosis and resistance to chemotherapy in many cancers (Liu and Jiang, 2006). CD44 can be found in collagen, fibronectin, fibrinogen, hyaluronic acid, FGF2, laminin, and other heparin-binding growth factors (Liu and Jiang, 2006; Naor et al., 2002). CD44 and its association to metastatic progression suggests its protagonism in the survival of disseminated cells, and this is enhanced through its upregulation by osteopontin (Desai et al., 2007). CD44 is increased in both SCLC and NSCLC and it correlates with survival (Le et al., 2006; Lee et al., 2005).

CD133 (Prominin-1)

Prominin-1 is a glycoprotein with five membrane-spanning domains initially identified in endothelial cells (Shmelkov et al., 2005). It is also found in brain, colonic, and pancreatic cancer-initiating stem cells (Vescovi et al., 2006). This protein, like CD44, has been linked to drug resistance and interestingly brain-tumor-derived CD133^{+}-sorted cell lines exhibit a unique gene expression profile (Beier et al., 2007). A recent study in humans from a histologically heterogeneous group of lung cancers analyzed SCLC and NSCLC tumors and identified a CD133^{+} subset of cells, which was able to produce lung tumor spheres with the ability to differentiate and produce tumors in vivo. This population of cells was found to be very similar to a rare subpopulation of CD133^{+} cells present in normal mouse lung, which undergoes significant expansion following airway injury after naphthalene treatment (Eramo et al., 2008). Expression of the ABC transporter responsible for the SP phenotype together with the embryonic stem cell markers, OCT4 and NANOG, was linked to chemoresistance in the CD133^{+}spheres (Eramo et al., 2008).

Miscellaneous Markers

In human SCLC cell lines, a small population of urokinase-type plasminogen activator (uPAR)-positive CD87 cells was identified. A subset of these cells showed increased clonogenic activity in vitro (Gutova et al., 2007). The presence of both uPAR^{+} and uPAR^{-} cells in the resultant colonies suggests a multilineage potential of those stem cell populations. However, when uPAR^{-} cells were isolated from the SCLC cell lines they lacked clonogenic activity. uPAR plays a role in cell adhesion and migration. Consequently, the fact that high levels of uPAR have been linked to various human cancers is not surprising (Romer et al., 2004).

Although for hematological, breast, and brain cancers many putative CSC markers have been readily identified, this, unfortunately, is not the case so far in potential human BASCs. The two cell surface markers used to identify mouse BASCs have proven to be inadequate in humans: Sca-1 does not have a human homolog, and CD34 staining does not correlate to the putative SP stem cells described in NSCLC (Ho et al., 2007). These results highlight the importance of further work to confirm whether a single, normal lung stem progenitor is sufficient to define the CSC populations in each subtype of lung cancer or whether it indicates multiple origins responsible for the cellularity of the cancers.

4 Conclusions

The growing field of pulmonary stem cells is resulting in useful study models that can be used to gain newer perspectives in the way cancer is investigated. These perspectives are certainly not devoid of controversy due to the many missing jigsaw pieces in the field. The discovery of stout cell surface markers will hopefully lead to the identification of putative human lung homeostatic and cancer stem cells as it has been done in other types of human cancer (Table 3). We have explored the role of the most important developmental pathways in the organogenesis of the bronchopulmonary tree to then highlight how these very same pathways can crosstalk through poorly understood mechanisms, to orchestrate the maintenance of the respiratory tree and its potential self-renewing stem cell populations during adulthood. Because of the large number of genes in these signaling transduction pathways and their specific functions in differing physiological and pathological contexts, a considerable proportion of developmental fates during the life of an animal are controlled by the action of these signals on stem cells and their specific niches or microenvironments. We have increasing evidence that the adequate function of stem cells is dependent on their niches, and these in turn are complex microenvironments composed of the extracellular matrix and various other cellular and non-cellular components that are also influenced by the paracrine actions of the developmental pathways described herewith (Table 3). All this taken together, it has been suggested that cancer is the result of a permanent state of injury repair, where the accumulation of oncogenic events may 'lock' activated previously homeostatic stem cells in a permanent abnormally driven mode that results in the transformation of these 'normal' stem cells into CSCs (Beachy et al., 2004) (Fig. 2). Understanding the complexity of these processes will hopefully allow scientists and clinicians to translating knowledge about the signaling machinery into novel pharmacologically relevant therapies for human lung cancers.

Table 3 Some examples of the involvement of developmental signaling pathways in cancer stem cells

Pathway	Tumor	Stem cell	Authors
Wnt	Colorectal cancer	Intestinal epithelial stem cells	Korinek et al. (1998), van de Wetering et al. (2002)
	Lymphoblastic leukemia	Hematopoietic stem cells	Willert et al. (2003), Reya et al. (2003), Chung et al. (2002), Qiang et al. (2003)
	Medulloblastoma	Cerebellar granule cell progenitors	Zurawel et al. (1998), Cui et al. (1998)
Shh	Glioma	Neural stem cells	Lai et al. (2003), Ruiz i Altaba et al. (2002)
	Medulloblastoma	Cerebellar granule cell progenitors	Wechsler-Reya and Scott (1999), Raffel et al. (1997), Pietsch et al. (1997), Goodrich et al. (1997)
Notch	Lymphoblastic leukemia	Hematopoietic stem cells	Pear et al. (1996), Varnum-Finney et al. (2000)
	Breast cancer	Mammary epithelial stem cells	Chepko and Dickson (2003), Jhappan et al. (1992)

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Cancer Stem Cells in Colorectal Cancer

Louis Vermeulen, Jan Paul Medema, James C.H. Hardwick, and Gijs R. van den Brink

Abstract Colorectal cancer is the second most common cause of cancer death in the Western world. Due to the high prevalence of colorectal cancer and precancerous lesions, many colonoscopies, and the resulting wide availability of tissue samples, extensive knowledge is available on the stepwise process that leads to colorectal cancer. Most colorectal cancers develop from slowly growing non-invasive adenomas that take many years to grow from a single mutant crypt to an adenoma that can reach several centimeters in size before acquiring invasive characteristics. In this chapter we will discuss some of the early histopathological events in the development of colorectal cancer and try to reconcile these data with the concept of the tumor-initiating or cancer stem cell. We will show that there are many similarities between the mechanism of stem cell expansion during intestinal growth and repair and deregulated clonal expansion of stem cells in an adenoma. We argue that it is important to realize the fact that most known genetic alterations in colorectal cancer development are involved in adenoma formation and are therefore involved in clonal growth and not invasiveness. It would therefore be helpful to distinguish adenoma stem cells from carcinoma stem cells. We will then discuss the available data on the isolation and behavior of colorectal cancer stem cells.

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1 Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death in the Western world with an estimated worldwide incidence of almost 1,000,000 per year (Mathers and Boschi-Pinto, 2006). This high prevalence and the easy accessibility of the colon for tissue sampling have contributed to the fact that CRC is one of the best studied malignancies. The development of a CRC involves a histopathological sequence of events caused by the stepwise genetic and epigenetic alteration of the expression of oncogenes and tumor suppressor genes. This succession of events has been dubbed the adenoma-carcinoma sequence (Fearon and Vogelstein, 1990). How these genetic changes drive the clonal proliferation of mutant colonic epithelial cells in adenomas and later in carcinomas remains an area of intense investigation. New insights from the up-and-coming field of cancer stem cell research may help to clarify how different oncogenes and tumor suppressors regulate clonal growth in the adenoma to carcinoma sequence.

The cancer stem cell model of colon carcinomas views a CRC as a hierarchical organized entity with a rare population of cancer stem cells driving tumor growth and progression (Vermeulen et al., 2008). Cancer stem cells can both expand in a clonal manner to generate more cancer stem cells and generate the more differentiated cells present in a CRC. It is believed that those differentiated cells have lost the potential to self-renew and subsequently the capacity to promote tumor growth in the long run. This new concept in colon-cancer biology has widespread consequences for the way we view this malignancy with respect to tumor initiation and progression of the disease and for the understanding of the mode of action of currently existing therapies for CRC and the design of future therapeutic modalities. Although useful when considering the biology of a colorectal cancer, the term colon-cancer stem cell unfortunately cannot be used for the adenomas that precede the cancer stage. Most colonic clonal growths

(neoplasms) are initially adenomas and only rarely undergo malignant conversion, a process that is typically estimated to take at least 10 years. In this chapter we therefore propose to distinguish adenoma stem cells from carcinoma stem cells and consider the term adenoma to carcinoma stem cell transition. We will give an outline of an adenoma to cancer stem cell model for colorectal cancer. We will discuss the methods and markers that are used to identify them and the relation between normal colon stem cells and colon-cancer stem cells.

2 Normal Colon Stem Cells

The colonic epithelial layer is characterized by its high turnover rate and is organized in functional units called crypts of Lieberkühn. Stem cells at the base of the crypt generate a population of transit-amplifying cells that fill the lower part of the crypt and divide several times before giving rise to the differentiated goblet cells, endocrine cells, and enterocytes. Crypts in both mice and humans are populated in a clonal manner (Winton et al., 1988; Novelli et al., 1996). The differentiated cells migrate upward and fill the upper part of the crypt and the intercrypt tables before being shed into the lumen of the gut. Colonic epithelial cells show a very high rate of renewal: the entire epithelial layer is replaced every 5–14 days depending on the species. To maintain homeostasis in such a rapidly renewing system, the control of cell fate in colon epithelium must be very tightly regulated. It is therefore no surprise that signal transduction pathways important for the development of the intestine such as the Wnt, Notch, TGFβ family and Hedgehog pathways regulate homeostasis of this highly dynamic system (van den Brink and Offerhaus, 2007).

2.1 Identification of Colonic Stem Cells

Until recently no simple reliable markers were available for the identification of colonic epithelial stem cells. Some researchers have used long-term DNA label retention to identify the stem cell compartment (Potten et al., 1997; Table 1). The idea behind those studies is that stem cells divide relatively infrequently while the more rapidly dividing progenitor cells dilute out the DNA label after the labeling pulse is given. Alternatively BrdU could be retained because stem cells would segregate their chromosomes asymmetrically to protect one strand against replication-induced somatic mutations (the so-called immortal strand hypothesis; Cairns, 2006). It has been difficult to prove that BrdU-retaining cells are really stem cells due to a lack of universally accepted stem cell markers in most organs. Recently, however, no evidence was found for label retention in hematopoietic stem cells (HSCs) that can be purified using well-characterized markers (Kiel et al., 2007). The authors demonstrated that HSCs are not slow-cycling cells but enter the cell cycle at a rate of 6% per day in mice. If we infer from this study that BrdU label retention may not be a reliable method to identify colonic stem cells, then the identification of colonic stem cells was

Table 1 Putative markers of normal colon stem cells

Markers	Function	Publication
Lgr5 (GPR49)	Unknown	Barker et al. (2007)
Musashi-1 (Msi1)	RNA binding protein possibly involved in asymmetrical cell division	Potten et al. (2003)
Label retention (BrdU)	–	Potten et al. (1997)

impossible until recently. Although Wnt signaling probably plays an important role in stem cell homeostasis in the small intestine and colon, stem cells cannot be marked using nuclear β-catenin staining (a read-out for active Wnt signaling, see below). Nuclear β-catenin also marks the transit-amplifying cells and in the small intestine it would mark the differentiated Paneth cells that are located at the crypt base (van Es et al., 2005a). Recently, however, Van der Flier et al. (2007) examined the expression of a large number of Wnt target genes in the small intestine by in situ hybridization and noted that Wnt targets could be categorized in three different expression patterns. The first pattern comprised most Wnt target genes ($\sim$80%) and showed expression in the transit-amplifying cells. The second pattern involved genes expressed in Paneth cells at the base of the small intestinal crypts. The third pattern was more unexpected and was characterized by expression by a few cells at the crypt base that were not Paneth cells. The same group has subsequently shown that *Lgr5* (*Gpr49*), one of the genes expressed in this pattern, marks the so-called crypt base columnar cells and may indeed be an intestinal stem cell marker, as lineage tracing experiments have demonstrated that *Lgr5*-expressing cells could generate all cell lineages (Barker et al., 2007). Interestingly, and in accordance with the life cycle of hematopoietic stem cells, it was shown that crypt base columnar cells are not slow-cycling cells but in contrast cycle approximately once per day. However, an important question still remains to be answered as the lineage tracing experiments showed that marked cells populated only part of the crypt. The marked $Lgr5^+$ cell did not therefore populate the whole crypt in a clonal manner as stem cells are believed to do. Does it simply take more time for the progeny of the traced $Lgr5^+$ cell to take up all the cell positions in a crypt or is the $Lgr5^+$ cell a cell that is downstream of a single dominant stem cell that generates a few different $Lgr5^+$ cells in a clonal manner? In conclusion, crypt base columnar cells are the most likely stem cells of the small intestine, and *Lgr5* seems to be an intestinal stem cell marker but much experimental work remains to further characterize these cells.

2.2 Morphogenetic Pathways Regulate Colonic Epithelial Cell Fate

2.2.1 Wnt Signaling

Wnt are small secreted glycoproteins that signal through a complex of a Frizzled receptor and a lipoprotein receptor-related protein (Clevers, 2006).

Classical or canonical Wnt signaling is regulated through control of the cellular localization of β-catenin. In the absence of Wnt ligand, β-catenin is continuously degraded in the cytoplasm by a complex of proteins consisting of Axin, GSK3β, and APC. This complex phosphorylates β-catenin, marking it for proteasomal degradation. Upon binding of Wnt ligands to their receptors, Axin is recruited to the membrane, resulting in inactivation of the β-catenin degradation complex, accumulation of β-catenin in the cytoplasm, and eventually translocation to the nucleus (Clevers, 2006). There β-catenin associates with members of the TCF/LEF family of transcription factors and induces transcription of a variety of target genes. Wnts and their antagonists are expressed in a complex pattern by both epithelial and stromal cells along the entire length of the crypt (Gregorieff et al., 2005). Wnt signaling through β-catenin normally occurs only in the cells at the base of the crypt as these cells show both nuclear localization of β-catenin and expression of β-catenin-TCF target genes. In the small intestine these cells include mature Paneth cells. Most Wnt target genes (approximately 80%) are expressed in the transit-amplifying precursor cells (Van der Flier et al., 2007) and it has been shown that this β-catenin-TCF induced transcriptional program is required for the specification of most aspects of the precursor cell phenotype (Van de Wetering et al., 2002). It is believed that upon migration of the cells toward the top of the crypt the level of canonical Wnt signaling decreases resulting in a loss of the precursor cell phenotype and induction of cellular differentiation. It has been shown that *Tcf4* is required for stem cell maintenance in the developing small intestine (Korinek et al., 1998), and mice that express the Wnt antagonist Dkk1 behind an intestinal epithelial-specific promoter fail to maintain their precursor cells during development of the intestine. It has been established that systemic expression of Dkk1 using a viral delivery system (Kuhnert et al., 2004) or conditional loss of β-catenin in the small intestinal epithelium of adult mice (Ireland et al., 2004) resulted in crypt loss. In conclusion, Wnt signaling seems to be the driving force of stem and precursor cell fate in the intestine although most of the experiments that have demonstrated this have been performed in the small intestine, an organ in which carcinogenesis is extremely rare in humans.

2.2.2 Notch Signaling

The Notch signaling pathway is a critical regulator of stem cell/precursor cell fate regulation and cell lineage specification. Notch interacts with its ligands of the Delta-like/Jagged family. As these are transmembrane proteins, Notch signaling is active in direct cell-to-cell contact. The classical example from *Drosophila* is the role of Notch signaling in lateral inhibition, allowing asymmetrical development of similar cells (such as a stem cell and its daughter cell). In the fly, Notch signaling suppresses Delta expression. Cell fate is therefore determined through feedback amplification of relatively small differences in Notch expression between both cells and one cell will end up as 'Notch high' whereas the other will be 'Delta high.' Activation of Notch receptors by

membrane-bound Delta on adjacent cells results in cleavage of the Notch intracellular domain by γ-secretase. The intracellular domain translocates to the nucleus where it directs transcriptional activity. Notch signaling in the 'Notch high' cell induces expression of Hes1, a transcriptional repressor. In many models the expression of this transcriptional repressor is important for the maintenance of the precursor cell/stem cell phenotype. This also seems to be the case in intestinal precursor cells, as inhibition of the Notch signaling cascade in mice by use of a γ-secretase inhibitor results in halted crypt proliferation and drives precursor cell descendants prematurely into the goblet cell lineage (van Es et al., 2005b). Intestinal restricted expression of a constitutive active form of the mouse Notch1 receptor in a mouse model results in an opposite phenotype, with expansion of the stem cell/transit-amplifying cell pool and inhibition of cell differentiation (Fre et al., 2005).

2.2.3 TGFβ Family Signaling

The TGFβ family of proteins includes the bone morphogenetic proteins (BMPs) and Activins. Members of the TGFβ family bind to a heterodimeric complex of a type I and type II receptor, resulting in the phosphorylation and subsequent activation of a receptor-regulated SMAD (SMAD2 and 3 for the TGFβ and Activin receptor complexes and SMAD1, 5, and 8 for the BMP receptor complex). These activated SMADs then form a complex with the co-SMAD SMAD4. The SMAD dimer translocates to the nucleus where it binds specific DNA sequences and directly modulates transcription. TGFβ1, TGFβ2, and TGFβ3 are all expressed in the adult colon. TGFβ1 is expressed by the differentiated enterocytes (Oshima et al., 1997), TGFβ2 by endocrine cells, and TGFβ3 by the cells at the base of the crypts (Dunker et al., 2002). Mostly based on in vitro experiments in colon-cancer cell lines it is often suggested that TGFβ signaling acts as a negative regulator of cellular proliferation in the colon. This is unlikely to be the major role in normal colonic epithelium in vivo as the type II TGFβ receptor is mainly expressed by the differentiated epithelial cells (Oshima et al., 1997). Indeed, mice heterozygous for either *Tgfβ2* or *Tgfβ3* display substantially reduced epithelial apoptosis whereas precursor cell proliferation is unaffected (Dunker et al., 2002). This corroborated earlier findings in *Tgfβ1*$^{-/-}$ mice that do not display increased proliferation either (Engle et al., 1999).

A variety of BMPs and their antagonists are expressed by epithelial and stromal cells in the colon (Li et al., 2007). Inhibitors of BMP signaling seem to predominate at the base of the crypt (Kosinski et al., 2007) resulting in a situation that favors BMP signaling in the cells at the top of the crypt, consistent with the expression of activated BMP-associated receptor SMADs in these cells (Hardwick et al., 2004). Similar to the role of TGFβ signaling in vivo, BMP signaling therefore regulates apoptosis of differentiated epithelial cells in vivo (Hardwick et al., 2004). In contrast to TGFβ signaling, however, the BMP pathway also seems to be a critical regulator of precursor cell behavior.

Mutations in the *BMP receptor type Ia* cause juvenile polyposis, an inherited polyposis syndrome (see later) (Howe et al., 2001). Experiments in mice have shown that *Bmp receptor 1a* mutations result in the expansion of epithelial precursor cells that form new crypt-like precursor cell compartments in the zone normally restricted to differentiated cells (He et al., 2004). An interaction between BMP and Wnt signaling is suggested by the accumulation of cells with nuclear β-catenin in these mice.

2.2.4 Hedgehog Signaling

Hedgehogs act through a complex receptor system. Hedgehogs bind the transmembrane receptor Patched. In the absence of Hedgehog, Patched restricts the activity of the signaling receptor Smoothened, probably through the control of the cellular localization of a second messenger molecule (Bijlsma et al., 2006). Upon binding of Patched by Hedgehog this negative regulation of Smoothened is relieved and signaling through Gli transcription factors ensues. The major Hedgehog in the colon is Indian Hedgehog (IHH). IHH is produced by colonocytes at the top of the crypt and stimulates differentiation of the enterocyte lineage (van den Brink, 2007). In addition, IHH antagonizes Wnt signaling, and a gradient of IHH from the top to the base of the crypt may be one of the factors that restrict active Wnt signaling to the bottom of the crypt.

2.3 Clonal Stem Cell Expansion in Colonic Epithelial Damage and Repair

Homeostasis of stem cell behavior is not only tightly regulated at the level of the single homeostatic crypt. Somehow the colonic epithelium is able to sense the number of stem cells present in a crypt or tissue zone encompassing several crypts and allows temporary lateral clonal expansion of stem cells during postnatal growth and in situations of damage (stem cell loss) and repair (stem cell replacement) (Leedham and Wright, 2008). This tightly controlled process involves the budding and subsequent fission of a single crypt to form two or more crypts from a single stem cell in a clonal manner (Greaves et al., 2006). The classical example of this manner of tissue regeneration is in inflammatory bowel disease where crypt fissioning is used as a pathological hallmark of the chronicity of the disease. A dramatic example of the impressive capacity for crypt renewal through lateral stem cell expansion was provided by Muncan et al. (2006) in c-myc conditional mutant mice. The strategy employed to conditionally knock-out c-myc resulted in a virtually complete loss of c-myc from the small intestine. The c-myc knock-out crypts became hypoplastic and retarded in growth. Somehow the few percent of remaining c-myc-positive crypts were able to repopulate the entire small intestine with c-myc-expressing crypts within 28 days. Unfortunately, the molecular mechanisms that restrict clonal stem cell expansion through crypt fissioning

and the mechanisms that temporarily allow such expansion during epithelial repair remain poorly studied and understood. Some understanding of the molecular machinery can, however, be extrapolated from the deregulation of the control of crypt fissioning that causes adenoma formation during the first steps of colorectal carcinogenesis (see next section).

3 Colorectal Carcinogenesis and Origin of the Colon-Cancer Stem Cell

As illustrated in the section above, a complex and only partially resolved interplay between different morphogenetic signal transduction routes regulates homeostasis of the colonic epithelial layer. The transition from this normal homeostatic system to the formation of an invasive carcinoma is a very slow, stepwise process that is estimated to take at least 10 years in humans. Colon cancers develop in the so-called adenoma to carcinoma sequence, a histopathologically recognizable series of events. Adenoma formation starts at the level of a single crypt that accumulates a larger-than-normal number of precursor cells that no longer occupy only the base of the crypt but lead to an expanded precursor cell zone more toward the top of the crypt and generate dysplastic, i.e., poorly polarized, and crowded epithelial cells (Leedham and Wright, 2008). The presence of abnormal proliferation and that of dysplasia are the hallmarks of an adenoma. The single crypt adenoma will subsequently expand laterally through the colonic epithelium through a process of crypt fission to form a very small or minute adenoma that now encompasses several crypts. Adenomas can continue to grow through crypt fission and possibly by mutant precursor cells leaving their own crypt and occupying neighboring crypts and the superficial epithelium. Adenomas classically appear macroscopically as exophytic polypoid growths within the colonic lumen that can reach several centimeters in size; however; they can also grow as flat lesions, the so-called flat and depressed adenomas, that can be hard to recognize during colonoscopy. It is important to realize that adenomas are in principle benign (non-invasive) lesions that are highly prevalent in the general population. The risk of malignant transformation of an adenoma correlates with adenoma size, the degree of dysplasia, and its morphology. Adenomas > 1 cm, with 'high-grade' dysplasia or villous histology, are believed to carry the highest risk of malignant transformation and are termed advanced adenomas. The prevalence of adenomas is difficult to estimate in the general population as only larger adenomas are reliably detected during colonoscopy. Even the prevalence of advanced adenomas is already around 6% in the population between 50 and 66 years of age at average risk for colorectal cancer (Regula et al., 2006). Given the fact that the prevalence of colorectal cancer is around 0.9% in the same population (Regula et al., 2006) most adenomas do not progress to cancer.

3.1 Clonal Stem or Precursor Cell Expansion Initiates Adenoma Formation

The possibility of lateral clonal expansion of stem cells through crypt fission during intestinal epithelial growth and repair needs to be tightly controlled to prevent the formation of clonal growths (neoplasms). It is therefore no surprise that mutations that initiate adenoma formation compromise pathways involved in the regulation of precursor crypt fissioning. The best example is probably the mutations in *APC* that result in adenoma formation in patients with familial adenomatous polyposis (FAP) and sporadic adenomas. Patients with FAP have a single functional *APC* allele; the reduced dosage of wild-type APC in normal crypts of the colon of these patients is sufficient to result in a substantially (19-fold) increased rate of crypt fissioning compared to normal (Wasan et al., 1998). Upon loss of the second *APC* allele in an epithelial cell, this mutant cell fills a crypt with its dysplastic progeny. Loss of APC not only causes dysplasia but also results in accumulation of mutated cells in the crypt. It is believed that when these precursor cells exceed a certain threshold the crypt starts to fission to form a second crypt. This is exactly what can be observed in minute adenomas. These small adenomas form by fissioning from the mutant 'mother-crypt' (see, e.g., Fig. 3B; van den Brink and Offerhaus, 2007). It is not known if the cell that gives rise to an adenoma is in fact a stem cell, a precursor cell, or even a differentiated cell that regains stem cell properties. Cells that drive adenoma growth are capable of formation of all cell lineages, self-renewal, and even clonal expansion but possess no malignant properties, i.e., are non-invasive. In this respect the term colon-cancer stem cell does not cover the long adenoma stage that precedes colorectal cancer and the term adenoma stem cell may be more apt to describe the cells that drive adenoma growth.

3.2 Identification of Initiators of Clonal Growth

Impressive insight has been obtained into the genes that are genetically or epigenetically modified during the formation of a colorectal cancer, especially with the sequencing of the entire genome of several different colorectal cancers (Wood et al., 2007; Sjoblom et al., 2006). Although we now have a long list of genes that are (epi)genetically modified during colorectal carcinogenesis, it is still difficult to say if these changes play a causal role and even more difficult to tell at what stage during the 10-year-long adenoma to carcinoma progression modification is required. As we have argued previously (van den Brink and Offerhaus, 2007) it is only in patients with inherited syndromes that predispose to colorectal cancer formation that the genetic lesion capable of initiating clonal growth is known with certainty. Mutations in the most frequently occurring genetic syndrome, the hereditary non-polyposis colorectal cancer (HNPCC) syndrome, are in mismatch repair genes. These mutations are not informative

for our understanding of the initiation of clonal growth as they are not initiators of clonal growth per se but cause genomic instability that subsequently results in other mutations that induce clonal growth.

3.2.1 APC Mutations and Adenoma Initiation

One of the major advances in our understanding of the initiation of clonal growth was made with the identification of *APC* as the gene mutated in patients with FAP and the subsequent demonstration that APC is required for the proper restriction of the activity of β-catenin-TCF/LEF signaling in what is called the canonical Wnt signaling pathway. It is now clear that Wnt signaling activity specifies colonic epithelial precursor cell phenotype and turnover. The mutation in *APC* is believed to result in unrestricted clonal expansion of the mutated cell due to the unrestricted Wnt pathway activity which maintains the cells in a precursor cell state, impedes their differentiation, and probably also their migration out of the crypt. The main remaining problem in the understanding of the effects of the APC mutation remains the fact that APC not only regulates the stability of β-catenin but is also involved in the regulation of chromosomal stability (Fodde et al., 2001). The *APC* mutation remains the only mutation of which certainty exists regarding its capacity to initiate clonal growth that results in adenoma formation in humans.

3.2.2 BMP and Receptor Tyrosine Kinase Signaling and Hamartoma Initiation

Mutations that initiate polyp growth have been identified in patients with genetic polyposis syndromes that do not result in adenoma formation but in hamartoma formation. Although hamartomas also show polypoid growth, they are distinct from adenomas in the sense that the epithelium on a hamartoma is non-dysplastic. In the general adult population hamartomas are very rare benign lesions that are not believed to progress to cancer. In patients with hamartoma syndromes areas of dysplasia can develop in some of the hamartomas, a process sometimes called adenomatous transformation. In contrast to sporadically occurring hamartomas, hamartomas of patients with hamartoma syndromes can progress to cancer (Giardiello et al., 1987; Brosens et al., 2007).

Mutations that initiate hamartomas may tell us little of the genetic lesions that initiate sporadic colorectal cancer but still give us a valuable insight into the mechanisms that regulate precursor cell homeostasis in the colon. As hamartomas are clonal growths, the mutations that cause hamartoma syndromes are very interesting from the perspective of our understanding of the molecular mechanisms that prevent clonal stem cell expansion. The genes mutated in these syndromes affect two morphogenetic pathways. Patients with juvenile polyposis have mutations in the *BMP receptor 1a* and its signaling mediator *SMAD4* (Howe et al., 1998, 2001). Patients with Cowden syndrome and Peutz-Jeghers syndrome have mutations in negative regulators of the PI3 kinase-Akt-mTOR signaling pathway, a critical pathway in receptor tyrosine kinase signaling.

Activity of PI3 kinase results in phosphorylation of the membrane lipid phosphatidylinositol 4,5-diphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 plays a critical role as a cell membrane anchor of AKT kinase, a central kinase in the suppression of apoptosis and induction of cellular growth and proliferation. AKT activation subsequently results in the activation of mammalian target of rapamycin (mTOR), a kinase involved in the regulation of protein synthesis and cell cycle progression. Cowden syndrome is caused by mutations that inactivate the phosphatase *PTEN* (Liaw et al., 1997). PTEN is the major antagonist of PI3 kinase as it is the phosphatase required for dephosphorylation of PIP3 to PIP2 and therefore the inactivation of PI3 kinase-mediated signaling. Patients with Peutz-Jeghers syndrome have mutations in the *LKB1* gene (Hemminki et al., 1998). LKB1 is a kinase that phosphorylates and activates cAMP-dependent kinase (AMPK), a critical negative regulator of mTOR.

In conclusion, three pathways have been identified that are critical regulators of clonal growth in colonic epithelium. The Wnt pathway stimulates clonal growth and is involved in adenoma stem cell formation that is relevant to our understanding of colorectal carcinogenesis. Formation of hamartoma stem cells is stimulated by the PI3 kinase signaling pathway and restricted by BMP signaling. Hamartoma stem cells behave differently from adenoma stem cells and their relevance to colorectal carcinogenesis is much less straightforward.

3.3 Adenoma to Carcinoma Transition

As described above adenomas are formed by accumulation of mutated adenoma stem cells and lateral expansion through crypt fissioning. Subsequent accumulation of (epi)genetic changes in the adenoma stem cells is probably responsible for the generation of a clone within the adenoma stem cell pool that has invasive (malignant) characteristics and causes the transition from adenoma to carcinoma. This mutant adenoma stem cell will be referred to as colon-cancer stem cell here. The generation of new mutations within the initial adenoma stem cell pool may explain why different degrees of dysplasia can be observed within an adenoma and small areas of invasiveness can be observed within early colorectal cancers that consist mostly of non-invasive adenomatous epithelium. Causality is much less clear in genetic lesions involved in adenoma to carcinoma transition than the *APC* mutations that are involved in adenoma initiation. One of the changes associated with adenoma to carcinoma progression is allelic loss of chromosome 17p, the minimal region being 17p13.1 which includes *p53* (Baker et al., 1989). When Baker et al. (1989) examined the remaining p53 allele in colon cancers with allelic loss of p53 they found that it was affected by point mutations in the remaining allele, suggesting that p53 was inactivated by two independent genomic 'hits.' While there have been several screens using different techniques to identify changes

associated with the adenoma to carcinoma transition (Agrawal et al., 2003), these remain associations: we must conclude that little progress has been made in identifying the precise genetic alterations involved in adenoma to carcinoma transition and a detailed discussion of this subject is therefore beyond the scope of this chapter.

4 Identification of Colon-Cancer Stem Cells

The framework described above has proven to be very fruitful in guiding oncological research in the understanding of the development of a colon carcinoma, but suggests a reductionist model of CRC as a monoclonal outgrowth of transformed cells. However, recent research shows that there is another layer of complexity involved in the generation and preservation of a colorectal malignancy. It has been suggested that only a subset of cells in a CRC has the capacity to fuel tumor growth in the long run and these cells have been dubbed colon-cancer stem cells. This would be in analogy to the situation in normal colon epithelium in which only a small number of stem cells generate the short-lived progenitor and differentiated cells. In addition, in various leukemias, including acute myeloid leukemia (AML), seminal studies by John Dick and co-workers identified a population of the so-called leukemia-initiating cells (LICs) that comprise the potential to transplant human AML into immuno-compromised mice (Bonnet and Dick, 1997; Lapidot et al., 1994). These initial findings in the field of cancer stem cell research were later expanded with other subtypes of leukemia and followed by identification of a tumor-initiating population in solid malignancies. In 2004, the first carcinoma in which a cancer stem cell population was described was breast carcinoma (Al Hajj et al., 2003). Very recently several studies point to the existence of a cancer stem cell population in colorectal cancer (CRC) as well (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Todaro et al., 2007a). In this section we will describe how these colon-cancer stem cells have been identified and characterized, and we will pay brief attention to the markers that are associated with the colon-cancer stem cell compartment. In addition we point out several caveats in the current available data and discuss crucial issues that need to be resolved before general acceptance of the cancer stem cell model for CRC can be expected.

4.1 Colon-Cancer Stem Cells: Definition and Methods

Normal stem cells are functionally defined by their ability to self-renew, i.e., give rise to more stem cells, and their capacity to spin-off more differentiated cells that fulfill specific functions inside an organ (Blau et al., 2001) as we have previously outlined for the normal colon epithelium here. For malignancies a consensus has recently been reached as to how to define the cancer stem cells in

an international meeting (Clarke et al., 2006). In analogy to normal stem cells, a cancer stem cell should be able to self-renew and generate the more differentiated cells present in a tumor. The practical translation of this consensus definition to date is the capacity of a cell population to initiate a phenocopy of the original malignancy upon injection into immuno-compromised mice (Clarke et al., 2006). Importantly, this newly established tumor should contain a functional cancer stem cell compartment. This is believed to address the issue of self-renewal and can be experimentally confirmed by sequential propagation of the malignancy into a secondary recipient. For example, in AML, purification of the $CD34^+$/$CD38^-$ fraction of cells results in enrichment for a population with the capacity to induce an AML, including a rare $CD34^+$/$CD38^-$ fraction, in SCID mice (Bonnet and Dick, 1997; Lapidot et al., 1994). The more differentiated $CD34^-$ or $CD38^+$ cells do not have this leukemia-initiating capacity. This suggests that the AML stem cells reside in the immature $CD34^+$/$CD38^-$ fraction of cells.

For solid malignancies purification of specific populations of cells is preceded by enzymatic digestion of the tumor. Enzymes such as collagenase and hyaluronidase are used to breakdown the extracellular matrix that surrounds the tumor cells. This procedure results in a single cell suspension that allows for selection of cells based on cell surface protein expression. Cells are purified by binding to antibodies and subsequent fluorescent-activated cell sorting (FACS) or magnetic bead separation.

4.2 *Colon-Cancer Stem Cell Markers*

Colorectal cancers are adenocarcinomas that form more or less differentiated glandular structures surrounded by variable amounts of stroma. The epithelial cells surrounding the crypts display clear heterogeneity in cell morphology and marker expression. This is somewhat similar to the situation in normal colonic epithelium where different cellular characteristics are associated with a variety of differentiated cell types. Moreover the heterogeneity present in normal colonic epithelium that results from a differential grade of differentiation can also be demonstrated in malignant transformed colon tissue. For example nuclear-localized β-catenin that is normally only detected at the crypt base and is thus associated with the stem and progenitor compartment is detected in a limited number of CRC cells (Fodde and Brabletz, 2007). In addition the colon precursor cell-associated protein Musashi-1 is present in a low number of cells in CRC tissue (Todaro et al., 2007b). So the question is whether these different populations of cells differ in their capacity to fuel tumor growth and their ability to transplant the malignancy to immune-deficient mice.

In the first reports describing isolation of a CRC cell population with the capacity to exclusively induce a phenocopy of the original primary human malignancy in mice, the surface marker CD133 was used for purification (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Todaro et al., 2007a; Fig 1).

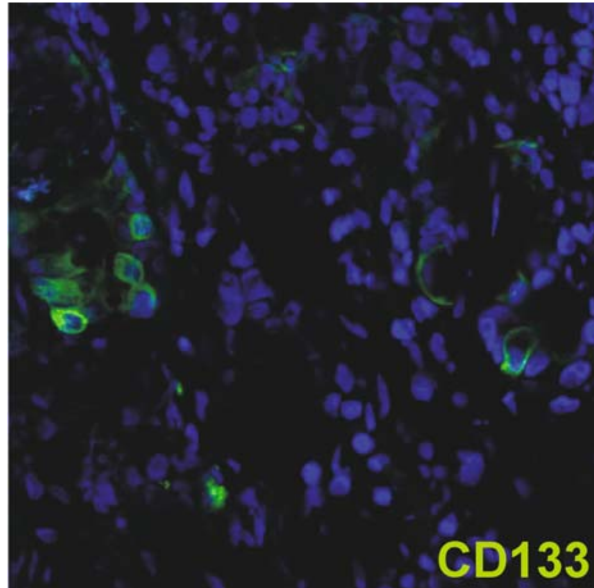

Fig. 1 Immuno-fluorescent staining of a primary human colon carcinoma shows a small population of $CD133^{+}$ epithelial cells

This glycosylated membrane protein is associated with immature cell types in a variety of tissues including brain and the hematopoietic system (Bhatia, 2001). Moreover, in a variety of brain tumors CD133 enriches for tumor-initiating cells (Singh et al., 2004). Low numbers of CD133-expressing cells have also recently been found in normal colon tissue (O'Brien et al., 2007). The function of the CD133 protein remains elusive to this point but is believed to be involved in plasma membrane physiology since its expression is highly restricted to membrane protrusions and co-localizes with membrane cholesterol (Mizrak et al., 2008). Severe retinal degeneration occurs in individuals homozygous for a frameshift mutation in the CD133 gene, suggesting a role for CD133 in photoreceptor disk morphogenesis (Maw et al., 2000).

In 2007 it was shown by three separate groups that as few as 100–3000 $CD133^{+}$ cells were able to initiate a new tumor while up to 10×10^{5}–6×10^{6} $CD133^{-}$ cells failed to grow a tumor upon subcutaneous injection (Ricci-Vitiani et al., 2007; Todaro et al., 2007a) or injection in the renal capsule (O'Brien et al., 2007). This indeed suggests that selecting the $CD133^{+}$ cell fraction results in enrichment for cells with tumor-initiating capacities, i.e., the cancer stem cells. Besides CD133, other markers have been implicated in describing the colon-cancer stem cell-containing population (Table 2). For example the $CD44^{+}$/ESA^{high} cells have also been reported to contain the cancer stem cell population (Dalerba et al., 2007). CD44 is an adhesion molecule and a Wnt target gene that is known to be expressed in the lower parts of the crypts in normal colon.

There are some indications that the putative normal colon precursor cell marker *Musashi-1* is also present in colon-cancer stem cells (Todaro et al., 2007b).

Table 2 Colon-cancer stem cell markers

Markers	Function	Percentage(%)	Publication
$CD133^{+}$	Unknown	0.2–20	O'Brien et al. (2007), Ricci-Vitiani et al. (2007), Todaro et al. (2007a)
$CD44^{+}/ESA^{high*}/CD166^{+}$	CD44; adhesion molecule, ESA; adhesion molecule, CD166 (ALCAM); adhesion molecule	0.2–58 (mean 11.8%)	Dalerba et al. (2007)

ESA epithelial-specific antigen

However, direct isolation of cells expressing this marker is not feasible at this point since this protein resides intracellularly. The frequency of tumor-initiating cells, even in the CD133-enriched population, is very low. The real cancer stem cell number is estimated to be, on average, 1 in every 262 $CD133^{+}$ cells (O'Brien et al., 2007). Moreover, clear overlap exists between the $CD133^{+}$ and $CD44^{+}/ESA^{high}$populations, indicating that a combination of these markers may further enrich for the colon-cancer stem cell population. This could indicate a hierarchy in stem cells and progenitor cells, as seen in the $CD34^{+}$ leukemia-initiating cell population in acute myeloid leukemia. Thus, additional markers are needed to purify the colon-cancer stem cell population further and to describe a possible progenitor phase.

4.3 *In Vivo Differentiation*

The tumors that arise after injection of the colon-cancer stem cell-containing cell population in mice are a phenocopy of the original malignancy. This means that the typical adenocarcinoma-like morphology is preserved and largely the same markers are expressed. To achieve this it is necessary that the injected cancer stem cells give rise to the more differentiated cells present in a CRC and, indeed, some cells within the xenografts do show expression of differentiation markers such as cytokeratin-20 (CK-20), CDX2, Muc-2, and Villin. These proteins are also present in the differentiated cells in normal colon crypts. Moreover, there is clear evidence for the production of mucin in these xenografts. This suggests that functional differentiation of colon-cancer stem cells also occurs. In addition, tumor cells are observed that express CD133, indicating that a colon-cancer stem cell population is preserved. These tumors are also able to propagate the tumor in a second round of transplantation and are still able to give rise to colon-cancer stem cell cultures (see later). In this way the two defining criteria for colon-cancer stem cells, self-renewal and generation of more differentiated cells, have been met.

The observation that the cancer stem cell-derived xenografts are heterogeneous with respect to both differentiation grade and differentiated cell phenotype argues that cancer stem cells remain sensitive to signals that guide differentiation. Indeed, nuclear β-catenin staining is only observed in a subset of cells in colorectal cancers. This shows that although the genetic defect that leads to Wnt signaling deregulation is present in all cells, maximal Wnt pathway activity only occurs in a subset of cells in the cancer (Fodde and Brabletz, 2007). Moreover, insights gained from the APCmin model for intestinal neoplasia show that in adenomas differentiation patterns can be influenced by interfering with the signals that guide normal differentiation in the intestine. When APCmin mice are subjected to γ-secretase inhibition, phenotypic changes are observed that are comparable to the response of normal intestinal epithelium, i.e., the induction of goblet cell differentiation (van Es et al., 2005b). This illustrates that colorectal cancers cannot simply be viewed as monoclonal expansions of malignantly transformed cells, but should be seen as heterogeneous, hierarchically organized tissues. Thus, although crucial homeostatic control is lost regarding extensive proliferation and invasive growth, other regulatory mechanisms that control cell differentiation are at least partially functional.

4.4 Caveats

To date all evidence that exists for the cancer stem cell model of malignancies is obtained using xenotransplantations (Hill, 2006; Vermeulen et al., 2008). In the case of CRC all available data suggesting the existence of colon-cancer stem cells involve complete tissue disruption to generate single cell solutions that can be sorted based on cell surface molecules. The next step involves injection of the cells into a non-orthotopic site, e.g., subcutaneously. Both experimental manipulations, the tissue disruption and the xenotransplantation, can cause significant biases in the interpretation of the data. For example, it is possible that $CD133^{+}$cells are better equipped than $CD133^{-}$ cells to survive the complete tissue disruption process or are better capable to grow out in mice (Hill, 2006; Vermeulen et al., 2008). It has been described, for example, that expression of the CD44 molecule has important implications for the engraftment capacity of cells in a wide variety of systems (Harada et al., 2001; Krause et al., 2006). Care has to be taken in the interpretation of the identity of a cancer stem cell marker. Is it a true marker in the sense that it is a read-out of a specific cellular state or, alternatively, are the markers themselves involved in the process studied, in this case engraftment and outgrowth of xenotransplants? In addition, the fact that the exclusive capacity to initiate a new tumor resides in the $CD133^{+}$ cell fraction does not necessarily tell us anything about the situation in an established CRC. So, while the data are in perfect accordance with a hierarchical organization of a CRC, other explanations are not convincingly ruled out at

this point. To elucidate the issues that remain, technically challenging mouse models need to be developed that allow for the specific killing or impairment of the proposed colon-cancer stem cells in a given tumor.

5 In Vitro Expansion of Colon-Cancer Stem Cells

To facilitate research on stem cells and recently on cancer stem cells, special cell culture methods have been developed. For colon-cancer stem cells, cells with an immature phenotype from a dissociated CRC are commonly cultured in specialized medium containing high amounts of growth factors such as endothelial growth factor (*EGF*) and basic fibroblast growth factor (*bFGF*) in the absence of serum. Moreover the cells are cultured in special treated plastics that do not allow for adhesion of the cells. In the resulting cultures the cells grow as spheres in suspension (Fig 2). The cells in these cultures express high amounts of the colon-cancer stem cell markers (including CD133 and CD44) and do not express differentiation-associated proteins (CK-20 and CDX2). As previously found in glioblastomas (Singh et al., 2004), it has been established that these culture techniques enrich for the cancer stem cells present in the primary malignancy (Vermeulen et al., submitted).

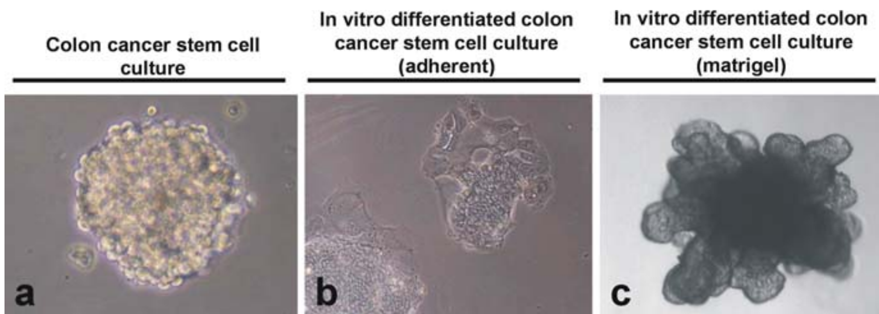

Fig. 2 (**a**) A typical spheroid in a colon-cancer stem cell culture. Spheroids are cultured in specially prepared cell culture flasks that do not allow the cells to adhere. High levels of bFGF and EGF are present in the culture medium. (**b**) Differentiated colon-cancer spheroid culture. Here cancer stem cell medium is removed and replaced by serum-containing medium. Moreover the cells are transferred to culture-treated plastics that do allow the cells to adhere. Cells lose the expression of stem cell marker CD133 and gain expression of cytokeratin-20. (**c**) Depicted is a spheroid that was transferred into matrigel and overlaid with serum-containing medium. The colon-cancer stem cells differentiate and start forming tubular structures

Interestingly these cancer stem cell cultures can be differentiated in vitro when subjected to differentiation-inducing conditions. For colon-cancer stem cells this is achieved by allowing them to adhere to tissue culture-treated plastics and providing them with fetal calf serum-containing medium. Moreover

3D-differentiation is reported using matrigel (a viscous mixture of extracellular matrix compounds). The in vitro differentiated cells lose expression of colon-cancer stem cell markers, such as CD133, and gain expression of differentiation markers (Fig. 2).

More importantly, the described colon-cancer stem cell cultures have the capacity to induce tumors in mice upon subcutaneous injection. These tumors exhibit a differentiated morphology together with preservation of a functional cancer stem cell compartment. This illustrates that the colon-cancer stem cell capacities have been retained during the period the cells were expanded in vitro. This is unique since normal CRC-derived cell lines, i.e., cultured in serum-containing medium on adherent plastics, can be tumorigenic upon subcutaneous injection in mice but result in an undifferentiated mass of cells (Weinberg, 2007; Fig 3). In glioblastoma it has been shown that the gene expression profiles of the cancer stem cell cultures, and derived xenografts, resemble the expression pattern of the original malignancy much more closely than do the classical adherent cell lines and their xenografts (Lee et al., 2006). In addition, the number of genetic alterations observed in the glioblastoma cancer stem cell was much lower than those observed in regular glioblastoma cell lines. Combined, these data suggest that cancer stem cell cultures are a culture system that is superior to traditional cancer cell lines irrespective of the cancer stem cell theory. However, care is warranted since the success rate of establishing a colon-cancer stem cell culture is limited (25–50%); evidently this results in a selection of malignancies, used for in vitro research, that contain a cancer stem cell compartment that can proliferate under the described culture conditions. This might indicate that the tumors that cannot be cultured efficiently in vitro contain cancer stem cells which are dependent on certain (growth) factors that are not present in the used medium. In this respect it is interesting to realize that

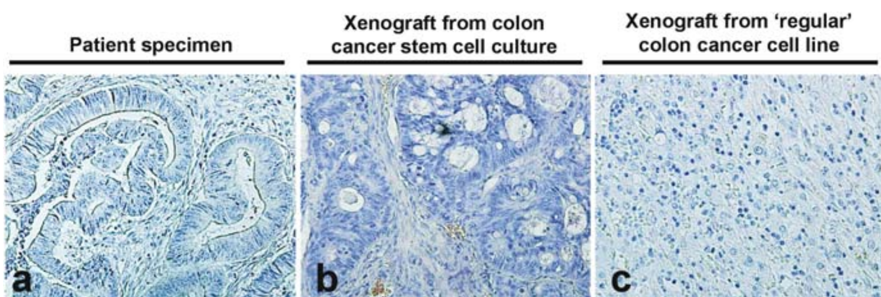

Fig. 3 (**a**) Typical histology of a colorectal adenocarcinoma. The epithelial cells form crypt-like structures surrounded by stromal cells. (**b**) Subcutaneous injection of colon-cancer spheroid cultures results in a tumor with a morphology that is similar to the original primary human malignancy. Note also the presence of stroma in the tumor. (**c**) Subcutaneous injection of a classical colon-cancer cell line, in this case HT29, results in an undifferentiated tumor mass. In those xenografts the typical morphology that characterizes colorectal cancers are mostly lost

it is easier to establish a colon-cancer stem cell culture from more advanced stage colorectal cancers, indicating that the cancer stem cells in these cancers may have become independent of a stem cell niche factor that is absent from the currently used culture media. However, this remains speculation to date.

The in vitro differentiated cells lose the capacity to initiate a carcinoma upon injection into mice. This exemplifies the intriguing consequence of the cancer stem cell model that genetically identical cells can have a completely diverse malignant potential. It is to date unknown if the more differentiated cells can revert back and adopt cancer stem cell capacities when exposed to de-differentiating signals. This seems unlikely in vitro since only the cancer stem cells characterized by CD133 expression can give rise to a colon-cancer stem cell culture. Moreover the experiments that involve direct cell sorting from CRCs show that if there is any de-differentiation present it is not a frequent event in these settings. However, this could be completely different in an established tumor that contains a putative colon-cancer stem cell niche.

6 Origin of the Colon-Cancer Stem Cell

Cancer stem cells have been defined as cells that (1) are able to self-renew and (2) give rise to the variety of differentiated cells present in a malignancy. However, it is not necessarily required that these cells are derived from a normal tissue-specific stem cell. In this respect the name 'cancer stem cell' can, and does, cause confusion and the alternative term 'tumor-initiating cell' might be better suited. However, the latter term implies that this cell was the cell that initiated the malignancy. This is not the case as there is clear evidence that the cancer stem cell compartment can undergo changes during the progression of the neoplasm. An additional problem with the term cancer stem cell is that it does not cover the adenoma stage during which clonal expansion begins. This problem can be solved by distinguishing the adenoma stem cell from the cancer stem cell and considering an adenoma to cancer stem cell model of colorectal cancer.

Although not necessary to fulfill the criteria to be called a cancer stem cell, there is some circumstantial evidence that in fact the colonic stem cells are the cells that acquire the various genetic hits to develop a CRC. The first argument is the long period a stem cell is believed to reside in the colonic crypts. Colonic stem cells are predicted to spend $\sim$20 years in the human body. The turnover of the more differentiated cells is believed to be completed every 7 days in the adult colon. So, in theory it is much more likely for the stem cells to be subject to acquiring malignancy-inducing genetic alterations. A good alternative, however, is for a genetic change to cause a more committed precursor cell to (re)gain stem cell-like properties. As differentiated cells are much more abundant in the colon, this could potentially compensate for the fact that the stem cells reside in the body much longer. It is therefore even possible that mutations in more

differentiated cells result in a delayed clearance or a reversion to a more immature phenotype. The cancer stem cell theory has only recently been revived due to technical advancements in sorting and culturing of specific cells. However, the hunt for the cell acquiring the right set of mutations to evolve into an adenoma and subsequently a carcinoma has been going on for several decades. In the genetic paradigm of colon-cancer research the term that is used to describe a stem cell origin of CRC is the 'bottom-up' hypothesis. This is used in contrast to the 'top-down' growth of colorectal adenomas that refers to a more differentiated cell type giving rise to the adenoma.

There are several lines of evidence suggesting a bottom-up histogenesis of colorectal adenomas. First is the observation of very early stage, monocryptal adenomas (Nakamura and Kino, 1984; Novelli et al., 1996). Novelli et al. (1996) convincingly show in a FAP patient that is also an XO/XY mosaic individual that these early lesions in the development of a colorectal cancer are monoclonal in origin since all monocryptal adenomas are completely either XO or XY. Moreover, in these micro adenomas, markers associated with the bottom of the crypts, where the stem cells reside, spread toward the crypt surface (Boman et al., 2004). This suggests an expansion of the crypt base cells, including the stem cells. Both intestinal adenomas in APCmin mice and human colorectal cancers show the presence of various types of differentiated cells such as goblet-like cells and neuroendocrine-like cells (Vermeulen et al., submitted; Pierce et al., 1977). This multilineage capacity of the cancer stem cells together with the apparent self-renewal capacities possibly reflects an immature cell of origin that has retained its stem cell-specific characteristics during malignant transformation. Alternatively a more differentiated cell has regained these traits in the process of conversion into a cancer stem cell. Conclusive evidence on this matter is anxiously awaited and can be expected to involve usage of technically challenging mouse models.

7 Cancer Stem Cells in Metastasis

In most patients that die from colorectal cancer, death is not caused by the primary tumor but by distant metastases. In colorectal cancer the predominant site for the development of metastasis is the liver. But metastases to lung, brain, and bone are also frequently found. This distribution of metastases is believed to be partly caused by the orientation of the draining vessels. In case of colorectal cancer the first capillary bed where the disseminated tumor cells could get stuck is in the liver. However, it is also proposed that tumor-specific characteristics are involved in the selection of the metastatic site. For example, certain growth factor dependencies of the malignancy can select for certain distant sites where successful outgrowth is possible.

An important prediction of the cancer stem cell model is that every metastasis is initiated by a cancer stem cell that succeeded in migrating out of the

primary tumor, evading the immune system, homing to and colonizing the distant organ. It is widely appreciated that formation of metastasis is an inefficient process. Estimations of cells that are shed into the vasculature are as high as 10^6 cells per gram of cancer tissue daily (Chang et al., 2000). Fortunately, this high frequency of tumor cell shedding into the vasculature does not result in an equally high rate of metastasis. Other, ethically controversial, experimental data show that upon autotransplantation of human tumor cells only a small fraction is capable of initiating an orthotopic tumor (Southam and Brunschwig, 1960).

These indications of the inefficiency of the metastatic process have been explained mainly in a genetic framework. It is believed that the additive genetic alterations required for successful metastasis are a relatively infrequent event. This might explain the low frequency of tumor cells capable of forming a metastasis. However, the new insights gained into cancer stem cell research may suggest an additional explanation. If only the cancer stem cells are in principle capable of founding a metastasis possibly this would explain the low numbers of metastasis-initiating cells. Furthermore this cancer stem cell model of metastasis is also supported by recent experimental data using gene expression micro arrays (Cardoso et al., 2007). It has been shown that the gene expression profile of the primary colorectal cancer is predictive for the occurrence of a metastasis later on in the disease. This suggests that the capacity to initiate a metastasis is possibly a feature of the dominant clone more than of a small fraction of cells that accumulated the right to mutations. Moreover, the gene expression profiles of metastasis closely resemble the expression profiles of primary colorectal cancers (Cardoso et al., 2007). These experimental data hint toward a model in which the small fraction of ‘migratory’ cancer stem cells is capable of invasion, extravasation, and establishment of a new tumor mass at a distant site (Brabletz et al., 2005). This metastasis then resembles the original malignancy in morphology, marker expression, and gene expression. Moreover this tumor contains again a cancer stem cell compartment from which colon-cancer stem cells can be isolated (O’Brien et al., 2007).

8 Consequences of the Cancer Stem Cell Model for Therapy

If the cancer stem cells are the only tumor cells capable of driving tumor progression and metastasis, every therapy should be aimed at successful eradication of the cancer stem cell compartment. However, evidence is accumulating that the population with cancer stem cell features is also more resistant to chemotherapeutic agents and radiotherapy (Al Hajj et al., 2004; Jordan et al., 2006). This putative chemo- and radio-resistance of the colon-cancer stem cells could possibly explain why such therapeutic interventions in CRC patients are of limited value. The identification of cancer stem cells as drivers of colon-cancer growth and the proposed resistance of these cells to existing

chemotherapeutic agents and radiotherapy have provided a novel direction of research for the development of future drugs.

8.1 Minimal Residual Disease

The cancer stem cell model could be an elegant explanation for a frequent clinical observation referred to as minimal residual disease (MRD) (Al Hajj et al., 2004; Jordan et al., 2006). The tumor mass responds very well to administered chemotherapy and no clinical signs of remaining tumor material are present, but after cessation of therapy the tumor rapidly re-grows. This effect is potentially caused by rapid tumor cell outgrowth from a small population of cancer stem cells that evaded the therapeutic regime and were capable of re-growing the original malignancy, while the bulk of the non-cancer stem cell tumor cells responded very well to the therapy. In a study using human colorectal cancer xenografts in mice a clear increase in the percentage of $CD133^{+}$ cells is observed under oxaliplatin treatment, providing experimental evidence for the proposed resistance of cancer stem cell compared to their differentiated counterparts (Todaro et al., 2007a).

We are only at the very beginning of understanding the nature of this relative chemo- and radio-resistance. The mechanisms involved in the cancer stem cell-specific therapy resistance, for other malignancies than colorectal cancer, are expression of multidrug resistance (MDR) proteins, differences in DNA repair mechanisms, differential sensitivity to apoptosis-inducing signals, and the possible quiescent state of the cancer stem cell compartment (Dean et al., 2005).

8.2 Chemoresistance of Colon-Cancer Stem Cells

It has recently been shown that IL-4 protects colon-cancer stem cells from the induction of apoptosis by chemotherapeutic agents in vitro and in vivo. Anti-IL-4 therapy resulted in in vivo sensitization of the colon-cancer stem cell to therapy with oxaliplatin (Todaro et al., 2007a). This illustrates how future therapeutic modalities might be developed; an agent that sensitizes or differentiates the cancer stem cells is combined with a chemotherapeutic agent that effectively kills the tumor cells, including the cancer stem cell compartment. This strategy prevents the cancer stem cells from surviving the therapy and effectively growing back the tumor.

8.3 Development of New Therapies

The cancer stem cell theory also implies that the way we approach the clinical testing of new drugs to treat malignancies has to be reconsidered. If indeed the

cancer stem cells are the cells responsible for long-term tumor growth and the formation of metastasis, then this is the clinically relevant population to target. At present new drugs are initially evaluated in late-stage patients and the most important parameter is tumor response. This leads to a selection of drugs that effectively target the bulk of tumor cells. This is a possible explanation for the so-called survival paradox, the fact that tumor response does not correlate very well with survival in cancer patients (Huff et al., 2006). Possibly promising new drugs that effectively target the cancer stem cell population are missed because of their limited effect on tumor volume in the short term, while on the contrary drugs are approved that reduce tumor mass but do not target the clinical-relevant population of cancer stem cells.

9 Synthesis

Stem cells in the normal colon occupy a niche at the bottom of the crypt. The expansion of these stem cells is highly regulated; under homeostatic conditions the stem cells and their transit-amplifying cells are tightly topographically restricted to their niche. However, stem cells can expand in a clonal manner under both physiological and pathophysiological conditions. Regulated clonal expansion of stem cells is observed during postnatal growth and in situations of damage and repair. Deregulated clonal expansion is the hallmark of the adenoma to carcinoma sequence that leads to colorectal cancer development. Both forms of clonal expansion are characterized by crypt fission. There is a close resemblance between the mechanism of crypt fission during intestinal repair and that observed in early-stage adenomas. The clonal expansion that is characteristic for the growth of early adenoma formation is therefore probably best seen as the unrestrained activation of a physiological repair mechanism. Research in the past decade has shown that colonic stem cell behavior is strictly controlled by the same morphogenetic signaling pathways that specify cellular fate during development. Mutations in these pathways lead to adenoma and hamartoma formation.

From histological examinations it is clear that an early adenoma is not a homogeneous mass of proliferating cells but a hierarchically organized lesion with proliferating cells and more or less differentiated derivatives. As an adenoma progresses to cancer and a cancer progressively becomes less differentiated this may become somewhat less straightforward but it is still clear from the pathology of most colorectal cancers that they do not look like homogeneous masses of cells but that clear heterogeneity in morphology and marker expression can be detected. Exciting new experimental data now show that cells in a colorectal cancer not only look heterogeneous but are, in fact, also functionally very distinct. Only a small population of immature cancer cells, dubbed cancer stem cells, can propagate a tumor in immuno-compromised mice whereas most cells in the tumor cannot. Furthermore, these cancer stem cells

lose this capability after being exposed to differentiation-inducing stimuli. Combined, this indicated that a stem cell-differentiated cell hierarchy is at least to some extent maintained in a colorectal cancer.

Of course the demonstration of the existence of cancer stem cells has far-reaching implications for our understanding of the (in)effectiveness of current treatment regimens and the authors hope that the possibility to propagate cancer stem cells in culture may advance the search for novel treatment regimes. A danger in the interpretation of the markers for cancer stem cells that are currently in use is that these markers may in fact identify cells that survive the experimental protocol for isolation and propagation of cancer stem cells. The actual cancer stem cells in vivo may therefore be (somewhat) distinct from those that we manage to propagate outside the original cancer. With the techniques to isolate and propagate cancer stem cells in place, progress in our understanding of their biology in the coming few years will undoubtedly be spectacular. Hopefully this will not only enlighten us on the understanding of colorectal cancer biology but also improve current treatment regimes and improve and prolong survival of advanced stage colorectal cancer patients.

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Cancer Stem Cells and Skin Cancer

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Abstract Skin cancers are the fastest growing type of cancer in the United States and represent the most commonly diagnosed malignancy, surpassing lung, breast, colorectal and prostate cancer. The epidermis is a multilayered epithelium that covers the skin providing a waterproof barrier that essentially controls the rate of water loss from the body. Recently, cancer stem cells (CSCs) are defined as cells with the capability of self-renewal, the potential to develop into any cell in the overall tumour population and to proliferate driving the continued expansion of the population of malignant cells. Thereby, the properties of tumour-initiating cells closely parallel to the features that define normal stem cells, i.e. asymmetric division. The molecular signature of skin stem cells and cancer stem cells is discussed.

According to the CSC model, clinical success depends largely on the CSC population either in quantitative terms such as the relative or absolute number of CSCs or qualitative aspects related to biological features of CSCs. The new pharmacological perspectives are also discussed.

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1 Introduction

Skin cancers are the fastest growing type of cancer in the United States and represent the most commonly diagnosed malignancy, surpassing lung, breast, colorectal and prostate cancer. In United States about 1 million of skin cancer

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occurred in 2007 (American Cancer Society, 2007). In Europe, the British Isles has been the highest rates of skin cancer in children and adolescents (de Vries et al., 2006). The most common types of skin cancer are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). In particular BCC of the skin is the most prevalent type of cancer affecting Caucasians (Daya-Grosjean and Couve-Privat, 2005; Holikova et al., 2004). However, the most dangerous type of skin cancer is malignant melanoma. This form of skin cancer can be fatal if it is not treated early but comprises only a small proportion of all skin cancers. In Europe a recent study showed that in the 19–20 year period of the study, the age standardized rate of malignant melanoma per million for adolescents in the British Isles increased from 5 to 29 (de Vries et al., 2006). In addition, in Europe as a whole it increased from 6 to 14 (de Vries et al., 2006).

1.1 Stem Cells in the Skin

The epidermis is a multilayered epithelium that covers the skin providing a waterproof barrier that essentially controls the rate of water loss from the body. In mammals, it comprises the interfollicular epidermis and the adexal structures, including the sebaceous glands and the hair follicles. The epidermis contains several types of cells, including keratinocytes (95%), melanocytes, Langerhans and Merkel cells. The different types of cells play an important role in maintaining the normal functions in the skin. The "stratum basale" (the basal layer) is the epidermis layer where most of the cells undergo rapid cell division in order to replenish the cells lost through terminal differentiation from the surface. Hair follicles are part of the skin epithelium, which is located in the dermis and associated with a sebaceous gland and a tiny bundle of muscle fibre called the arrector pili. Hair follicles are self-renewing structures that reconstitute themselves through the cycle comprising anagen (growing phase), catagen (regression phase) and telogen (resting phase). Melanocyte stem cells reside in the bulge region of the hair follicle (Nishimura et al., 2002; Amoh et al., 2005).

The terminally differentiated cells in all regions of the epidermis are continually shed from the skin and must be replaced throughout adult life. The replacement depends on the stem cells. These cells show an extensive self-renewal capacity and produce progeny that undergo terminal differentiation along the different epidermal lineages. The hierarchical model of cellular replacement was originally described by Gilbert and Lajtha (Gilbert and Lajtha, 1965) for haematopoietic bone marrow and, more recently, for cutaneous epithelium by Potten and Booth (Potten and Booth, 2002). In both models, stem cells are thought to be quiescent or to cycle slowly and to be protected within the tissue architecture. When stimulated, they should be capable of considerable proliferation and some of these cells are multipotential and capable of producing more than one differentiated lineage. Epidermis of mice was

demonstrated many years ago by Potter Laboratory that is organized into proliferative units (Allen and Potten, 1974; Potten, 1974). Accumulated evidences indicate that keratinocyte stem cells (KSCs) reside in the bulge area of the hair follicle both in rodent and human (Cotsarelis et al., 1990; Lyle et al., 1998; Morris and Potten, 1999; Taylor et al., 2000). However, there are also stem cells in the interfollicular epidermis and, potentially, in the sebaceous gland (Fig. 1). The stem cell progeny that are destined to terminally differentiate can first undergo a few rounds of divisions, during which time they are known as transit-amplifying cells (Niemann and Watt, 2002). Thereby, at present it is unclear whether transit-amplifying cells have multilineage differentiation potential or they are lineage-restricted.

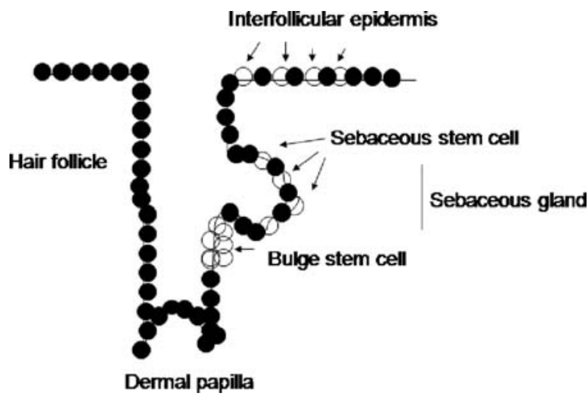

Fig. 1 The epidermis is a multilayered epithelium that covers the skin providing a waterproof barrier that essentially controls the rate of water loss from the body. The different types of cells play an important role in maintaining the normal functions in the skin. In this figure is shown the localization of stem cells in mammalian epidermis. The terminally differentiated cells in all regions of the epidermis are continually shed from the skin and must be replaced throughout adult life. The replacement depends on the stem cells. These cells show an extensive self-renewal capacity and produce progeny that undergo terminal differentiation along the different epidermal lineages. Stem cells are present at the follicle bulge, at the basal layer of the interfollicular epidermis. There are conflicting reports as to whether they are clustered (like as in the hair follicle) or distributed singly. There are a third stem cell population in the sebaceous gland. However, it is possible that the latter is maintained by bulge stem cells. Furthermore, there are transit-amplifying cells and cells that are withdrawn from the cell cycle and become committed to terminal differentiation

Molecular signature of living bulge cells was delineated and their biological behaviour in vitro and in vivo was studied (Ohyama et al., 2006; Tumbar et al., 2004; Morris et al., 2004; Blanpain et al., 2004). In particular, the molecule signatures of the mouse bulge cells were successfully obtained by two groups (Tumbar et al., 2004; Morris et al., 2004), identifying 57 overlapping genes upregulated including genes associated with growth arrest or proliferation and

differentiation such as Gas1 and tenascin C. Recent studies also demonstrated the crucial roles of Rac1 and Lhx2 for the maintenance of hair follicle stem cells (Benitah et al., 2005; Rhee et al., 2006). In particular, mouse bulge markers include CD34 keratin 15 (K15) and the β4 and β1 integrin subunits (Fuchs et al., 2004; Morris et al., 2004; Tumbar et al., 2004; Cotsarelis, 2006). Recently, the genes upregulated in the bulge of mouse and human hair follicles have been compared (Cotsarelis, 2006; Ohyama et al., 2006). K15 is a bulge marker in both species, but in human follicles CD34 is expressed below the bulge (Lyle et al., 1998). Laser capture microdissection and microarray analysis revealed a panel of cell surface markers for human bulge cells. CD200 and CD59 are upregulated in the bulge compared to other defined hair follicle regions, while CD24, CD34, CD71 and CD146 were downregulated (Ohyama, 2007). In particular, CD200 was preferentially distributed in the bulge area and represented the best positive surface marker for the human bulge cells (Ohyama, 2007). It is an interesting marker since it plays a role in the communication with the immune system (Cotsarelis, 2006; Ohyama et al., 2006). On the other hand, the expression of Dkk3 and Wif1 (Wnt-inhibitor factor 1) in the mouse and human bulge underscores the importance of genes that maintain stem cells in a nonproliferative state. Although integrin-mediated adhesion to the extracellular matrix negatively regulates terminal differentiation of cultured keratinocytes, epidermal deletion of integrins in vivo does not lead to a stem cell depletion phenotype too (Lopez-Rovira et al., 2005). In contrast, deletion of Rho family GTPase Rac1, which regulates cell adhesion and growth factor responsiveness, results in stem cell depletion and in the failure to maintain the interfollicular epidermis, hair follicles and sebaceous glands (Benitah et al., 2005). Rac1 plays an important role in the epidermis because Rho signalling suppresses differentiation of cultured keratinocytes as does the Rho effector citron kinase (CRIK). Rho and CRIK might act by affecting integrin and Notch signalling (Grossi et al., 2005). One way in which Rac1 maintains the stem cell compartment is by negative regulation of Myc. This occurs through PAK2 phosphorylation of Myc, which decreases Myc binding to Max and DNA (Benitah et al., 2005). In the skin epidermis, c-Myc is expressed in both the proliferative basal layer, which comprises stem-progenitor cells and transient-amplifying cells, and the bulge region that contains multipotent epidermal stem cells (Bull et al., 2002). Activation of Myc causes cells to exit from the epidermis stem cell compartment and stimulates differentiation into interfollicular epidermis and sebaceous glands. Another way, Myc acts by Miz1-dependent repression of cell adhesion genes, including the β6 and β1 integrin subunits (Gebhardt et al., 2006). More recently, Myc was demonstrated to trigger transit-amplifying divisions by inducing expression of growth promotion genes such as Misu (Frye and Watt 2006). Although overexpression of Myc triggers terminal differentiation, ablating Myc from the epidermis results in insufficient expansion of the stem cell compartments (Zanet et al., 2005). Thereby, stem cells seem to require low level of Myc to proliferate and Myc-induced differentiation preventing uncontrolled proliferation. Transgenic mice that express c-Myc in the basal cells suffer from

problems in wound healing and die or have to be sacrificed in the first few weeks after birth due to skin ulceration caused by depletion of a functional stem cell pool (Wakel et al., 2001; Arnold and Watt, 2001). This might be due to a reduction in adhesive interactions between stem cells and the local environment allowing them to exit from their normal niche (Frye et al., 2003). Myc epidermal knockout mice (KO) are viable and their keratinocytes continue to cycle, but they display sever skin defects. In particular, keratinocyte cell size, growth and endoreplication are reduced as well as stem cell amplification is compromised (Zanet et al., 2005). Interestingly, this chapter suggests that stem cell turnover is higher in KO Myc mice and that their compartment might be reduced. Moreover, a consequence of forcing stem cells to divide is that the epidermis would age prematurely, in fact the KO Myc skin mice appeared atrophic and showed a slow regenerating capacity (Zanet et al., 2005). Ectopic expression of c-Myc also renders keratinocytes resistant to growth inhibition by TGFβ1 (Alexandrow et al., 1995).

Tert is another factor which promotes stem cell mobilization in the absence of changes in telomere length and beta-catenin stabilization which promotes the transition from quiescent stem cells to proliferating cells in the bulge (Lowry et al., 2005). When cells exit from the stem cell compartment, they undergo a few further rounds of division, during which time they are known as transit-amplifying cells. Thereafter, they undergo terminal differentiation along several distinct lineages, forming the interfollicular epidermis, sebaceous gland and hair follicle (Niemann and Watt, 2002).

Wnt signalling through β-catenin plays a pivotal role in controlling the various stem cell populations in the mammalian body (Reya and Clevers, 2005). Wnt ligands constitute a family of highly conserved secreted glycoproteins that activate a cascade of cellular signalling events known as Wnt/β-catenin signalling pathways (Wodarz and Nusse, 1998; Logan and Nusse, 2004). In the skin, Wnt/β-catenin signalling is important in cell fate determination, differentiation and morphogenesis (Fuchs et al., 2001; Niemann and Watt, 2002; Millar, 2002). Multiple Wnts and frizzled receptors are expressed in the skin in highly dynamic patterns at different stages of development and provide good candidates for mediating all the different effects attributed to the Wnt/beta-catenin signalling cascade (Reddy et al., 2004; Millar, 2002). In vivo studies analysing Wnt-responsive beta-galactosidase reporter in the skin revealed the strongest activity in matrix cells at the base of the growing hair follicle (DasGupta and Fuchs, 1999). In postnatal skin activated β-catenin was demonstrated to induce new hair follicle structures from the interfollicular epidermis, the sebaceous gland and the outer root sheath of existing hair follicles even when the β-catenin signal is temporally restricted (van Mater et al., 1998; Lo Celso et al., 2004). Remarkably, β-catenin-induced hair follicles compose a bulge region with expression of stem cell markers and dermal papillae and can undergo cycles of hair regeneration (Silva-Vargas et al., 2005). Thereby, Wnt/β-catenin signalling levels determine the cell fate and lineage commitment in the skin: strong activation of the pathway leads to stimulation of hair

morphogenesis whereas repression of signalling results in differentiation in sebocytes and interfollicular epidermis (Niemann and Watt, 2002). Different regions of the epidermis differ in their sensitivity to a given level of beta-catenin activation (Lowry et al., 2005). Furthermore, sustained activation of low levels of β-catenin results in mobilization of stem cells in the bulge (Lowry et al., 2005). Stem cell activation by low-level Wnt/beta-catenin signalling was reflected by precocious entry into anagen, the phase of new hair follicle growth. Shifting the activation state of the stem cell compartment occurred without gross perturbations of the stem cell niche as size and morphology and expression of stem cell markers of the bulge region remain unaffected. On the other hand, the gene ablation of β-catenin in the skin results in failure of the stem cell niche to express their characteristic marker molecules (Lowry, 2005; Huelsken et al., 2001). Two pathways intersect the Wnt pathway in epidermis, the Notch and vitamin D pathways. Notch 1 is an essential factor of postnatal hair follicle development and homeostasis (Vauclair et al., 2005). On the other hand, loss of mutation on vitamin D receptor is associated with hair loss in mice and humans (Shah et al., 2006).

In stem cell progeny, Wnt/beta-catenin signalling activates a set of genes associated with the proliferation of hair follicle keratinocytes such as Sonic Hedgehog (Shh), Patched (Ptc), bone morphogenic proteins (BMP 2/4), FoxN1 (Gat et al., 1998; Silva-Varges et al., 2005;). Pharmacological inhibition of Shh signalling in combination with moderate activation of beta-catenin, blocks de novo hair follicle formation (Silva-Vargas et al., 2005). Conversely, inhibition of Shh signalling improves morphogenesis in response to high levels of beta-catenin activation (Silva-Varges et al., 2005). Sox9 expression is dependent on Shh and deletion of Sox9 in the epidermis results in hair loss and failure to form a bulge (Vidal et al., 2005).

Involvement of Notch signalling in postdevelopmental stem cell system is best understood in the skin, particularly in the hair follicles. Notch1, Notch2 and Notch3 are expressed and differentially localized to various layers of the hair follicle (Powell et al., 1998; Pan et al., 2004). Notch signals do not affect the pattern of skin formation during embryogenesis. After birth, the first cycle of Notch-1 null mice shows a shortened anagen phase and premature entry into the catagen phase, and inactivation of Notch1 in adult mice results in almost complete hair loss followed by cyst formation. Thereby, Notch1 is essential for postnatal hair follicle development and homeostasis (Pan et al., 2004; Vauclair et al., 2005).

Notch over-activity in hair follicle cells also leads to abnormal hair formation (Lin et al., 2000; Uyttenadaele et al., 2004). In summary, Notch signals are likely to promote the selection of hair formation in bulge stem cells. Recently, Notch signalling acting through Hes 1 was demonstrated to play a crucial role in the survival of immature melanoblasts and melanocyte stem cells by preventing initiation of apoptosis (Moriyama et al., 2006).

Interestingly, p63 a homolog of the p53 tumour suppressor in skin development was revealed by two independent studies of p63-deficient mice. These mice lack stratified epidermis, producing a disorganized single-layered surface

epithelium that is negative for epidermal markers such as keratin (K) 5 and 14 (Yang et al., 1999; Millis et al., 1999). Furthermore, p51/p63 was recently demonstrated to maintain the immaturity of keratinocytes stem cells by inhibiting Notch1 activity (Okuyama et al., 2007).

2 Cancer Stem Cells and Stemness Signature in the Skin

Many studies performed over the past 30 to 40 years have shown that the characteristics of stem cell systems, the specific stem cell properties described for stem cells, are relevant to some forms of human cancer (Reya et al., 2001; Al-Hajj et al., 2004; Fialkow et al., 1967; Hamburger and Salamon, 1977). Biologically distinct and relatively rare population of tumour-initiating cells have been identified in cancers of the haematopoietic system, brain, breast, melanoma and other tumours (Lapidot et al., 1994; Bonnet and Dick, 1997; Singh et al., 2004; Monzani et al., 2007; Al Hajj et al., 2003; Ignatova et al., 2002; Singh et al., 2003; Galli et al., 2004). Cancer stem cells are defined as cells with the capability of self-renewal, the potential to develop into any cell in the overall tumour population and to proliferate driving the continued expansion of the population of malignant cells (Fig. 2). Thereby, the properties of tumour-initiating cells closely parallel the three features that define normal stem cells. In fact both cells show the capability of asymmetric division into another stem cell and one progenitor cell that further differentiate into mature progeny comprising the adult tissue (Lajthe, 1979).

Regarding the origin of cancer stem cells, there are several lines of evidences indicating that cancer stem cells can arise from mutated progenitor cells (Jamieson et al., 2004; Cozzio et al., 2003; Huntly et al., 2004; Krivstov et al., 2006). However, they might derive not only from stem cells pools but also from differentiated cells that undergo transdifferentiation processes (Fig. 2). Although many tumours contain cells that display stem cell-like features, the identity of the normal cells that acquire the first genetic hits leading to the cancer-initiating cells remains elusive (Perez-Osada and Balmain, 2003). The discovery that bone marrow-derived stem cells home to sites of tissue damage opens up another possibility for the origin of cancer stem cell (Borue et al., 2004; Brittan et al., 2005) (Fig. 2).

In the stem cell model the cancer disruption of genes involved in the regulation of stem cell self-renewal seems to be important. It is thought that the environment or the niche surrounding stem cells provides signals necessary for the stem cells to continue to self-renew and upon exit from this niche the stem cells begin to undergo differentiation (Spradling et al., 2001). The mechanism by which stem cells decide to either remain in the niche or leave it could be a major factor in the balancing act between stem cell self-renewal and differentiation (Wallenfang and Matunis, 2003). In this connection, melanocyte growth is under the control of keratinocytes and melanoma seems to escape from such a control through different mechanisms such as downregulation of receptors

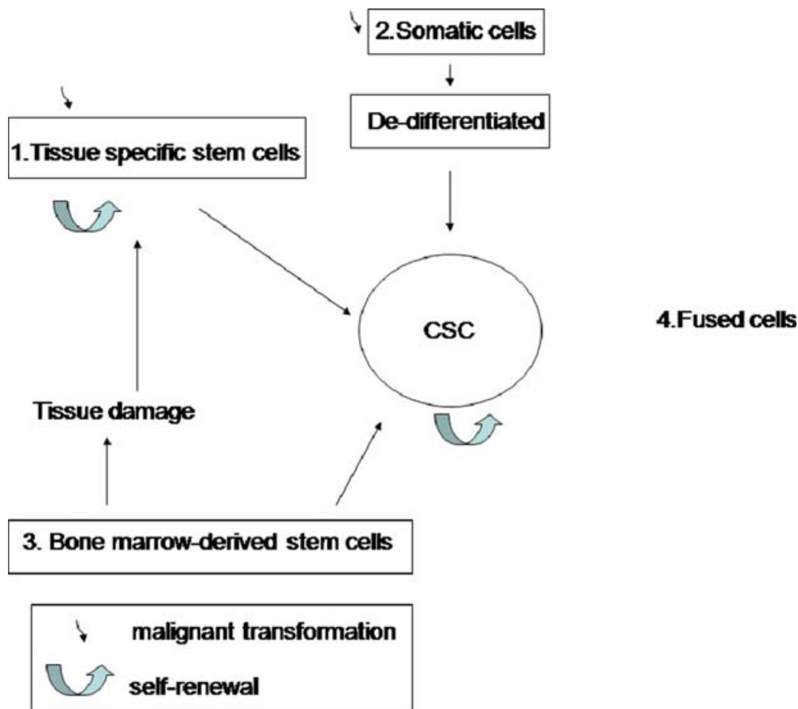

Fig. 2 Normal tissue stem cells are defined by three common proprieties: first, the presence of an extensive capacity for self-renewal that allows maintenance of the undifferentiated stem cell pool; second, strict regulation of stem cell number: the asymmetric division into another stem cell and one progenitor cell that further differentiates into the mature progeny comprising the adult tissue. Third the capacity to differentiate into particular mature cell types. Therefore, it is possible that a minor subversion of normal stem cell might be sufficient to create a malignant condition (**1**). Like normal stem cells, CSCs can self-renew and give rise to heterogeneous population of daughter cells and proliferate extensively. On the other hand, mutation in somatic cells might re-program these to CSCs (**2**). Bone-marrow-derived CD34+ stem cells can migrate to the site of tissue damage where they become tissue-specific stem cells and are prone to malignant transformation (**4**). Finally, stem cell can fuse with somatic cells and in this way found a cancer stem cell. The transforming event could occur in the same stem cell, the somatic cells or the fused cell. Self-renewal is indicated by a *curved arrow*

(i.e. E-cadherin, P-cadherin and desmoglein), upregulation of receptors and signalling molecules important for melanoma cellmelanoma cell and melanoma cell–fibroblast interactions (i.e. N-cadherin, zanula occludens protein -1) and deregulation of morphogenesis such as Notch receptors and their ligands. The investigation of normal melanocyte homeostasis might help us to define how melanoma and, in particular, melanoma cancer stem cells escape the microenvironment created by epidermal keratinocytes and how they develop new cellular partners in fibroblasts and endothelial cells which support their growth and invasion (Haas and Herlyn, 2005).

The stemness is conceivably maintained through deregulation of pathways such as Bmi-1, Notch, Wnt and Sonic Hedgehog (Shh). Recent evidence suggests an additional existence of distinct self-renewal mechanisms between normal stem cells and CSC involving the tumour suppressor protein Pten (Yilmaz et al., 2006). Both Wnt signalling and Shh signalling are inappropriately activated in epidermal tumours (Niemann and Watt, 2002; Owens and Watt, 2003; Fuchs et al., 2004; Hutchin et al., 2005). Uncontrolled activation of these pathways may result in specific cancers, possibly as an attempt to recapitulate normal embryonic organogenesis (Molofsky et al., 2004; Ruiz et al., 2004). Aberrant Notch signalling has been detected in several cancers and has recently strongly connected to T-cell acute lymphoblastic leukaemia (Grabber et al., 2006). Aberrant activation of the Wnt pathway has been found in a variety of human tumours and is strongly associated with colorectal cancer (Taipale and Beachy, 2001). Increased Hedgehog signalling has been linked not only to small subset of tumours in the brain, skin and muscle but recently also to cancers in the lungs, gastrointestinal tract and pancreas (Pasca di Magliano and Hebrok, 2003). Bmi-1, a transcription repressor of Ink4a/Arf locus (Jacobs et al., 1999) which encodes two separate tumour suppressor genes p16 and p14, is an oncogene that is found overexpressed in several human cancers (Valk-Lingbreek et al., 2004) like, for instance, in the majority of medulloblastomas (Leung et al., 2004; Marino, 2005). More recently, Bmi-1 expression was demonstrated to be associated with an increased risk of melanoma metastasis (Mihic-Probst et al., 2007). Finally, activation of Myc, a classical proto-oncogene, was demonstrated not only to promote growth and proliferation but also to induce highly invasive tumours in a transgenic model of pancreatic tumorigenesis using the insulin promoter (Pelengaris et al., 2002). In addition, c-myc is closely correlated with the progression from the in situ to the invasive stage in human breast carcinomas (Robanus-Mandag et al., 2003). Therefore, the disruption of c-Myc through Miz1 could contribute to the genesis of skin tumours affecting or contributing to the development of cancer stem cells. More recently, CD133-positive cells were demonstrated to occur in melanoma biopsy (Monzani et al., 2007). Furthermore, a recent paper showed an increased expression of CD166, CD133 and nestin during melanoma progression (Klein et al., 2007).

3 Pharmacological Perspectives

Cancer chemotherapeutic efficacy is frequently impaired by either intrinsic or acquired tumour resistance to multiple, structurally unrelated therapeutic drugs with different mechanisms of action, a phenomenon termed multidrug resistance (MDR). MDR can result from several distinct mechanisms, including alterations of tumour cell cycle checkpoints, impairment of tumour apoptotic pathways, repair of damaged cellular targets and reduced drug accumulation in tumour cells (Gottesman et al., 2002).

The clinical significance of CSC population remains unclear. According to CSC model, clinical success depends largely on the CSC population either in quantitative terms such as the relative or absolute number of CSCs or qualitative aspects related to biological features of CSCs. So far there are few data addressing this question: a paper showing a higher percentage of CD34–CD38 blasts correlated with a poorer survival in acute myeloid form of leukaemia (AML) (van Rhenen et al., 2005); another study showed that the percentage of CD133-expressing cells in malignant brain tumours correlated with the rate of tumour formation when implanted in immunodeficient mice (Bao et al., 2006). On the other hand, certain natural properties of CSCs are likely to increase their resistance to standard chemotherapy agents. In this connection, an intriguing possibility is that CSCs express high levels of specific ABC drug transporters (Fig. 3). Recently, ABCB5 was demonstrated to be expressed by a subset of human melanoma cells (Frank et al., 2003). In physiological progenitor cells ABCB5 functions to maintain membrane hyperpolarization, thereby serving as a negative regulator of cell fusion of the expressing progenitor subset and as a consequence of culture growth and differentiation (Frank et al., 2003).

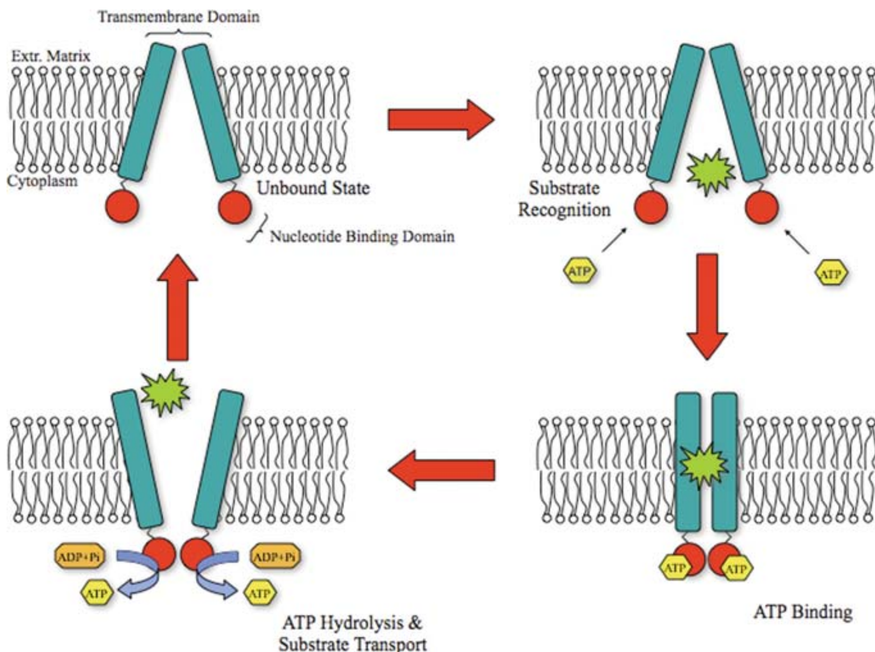

Fig. 3 ABC transporter molecules are responsible for ATP-dependent caring substances (*green*) back and forth across the inner membranes of cells. In the inner part of the transporter the hydrolysis of ATP through the ATP-binding domain induces conformational changes of the ABC transporter. The ABC transporter is a large family comprising ABCG2 and ABCB5 molecules, both expressed in human melanoma cancer stem cells

Furthermore, more recently ABCB5 was demonstrated to mediate doxorubicin transport and chemoresistance in a subset of human malignant melanoma (Frank et al., 2005). Furthermore, in melanoma a subpopulation of CD133/ABCG2+ cells were demonstrated (Monzani et al., 2007) according to other tumours (Zhou et al., 2001; Doyle and Ross, 2003). More recently, systemic administration of a monoclonal antibody directed against ABCB5 was demonstrated to induce antibody-dependent cell-mediated cytotoxicity in ABCB5+ cells exerting tumour-inhibitory effects (Schatton et al., 2008).

Thereby, it is clear that if the tumour is driven by CSCs, a radical change in the treatment of tumour should arise. In particular, new diagnostic and therapeutic targets expressed by stem cells should be identified. Clearing the CSCs should cure the disease since the remaining cells have limited proliferative capabilities. However, targeting CSCs is problematic since CSCs share many molecular factors with normal stem cells (Galmozzi et al., 2006) such as PTEN, Bmi-1. Thus targeting such a modulator of CSCs is likely to increase side effects resulting from stem cell loss. Therefore, the similarities between CSCs to the adult tissue stem cell seriously hinder systemic cytotoxic therapies.

To bypass all these problems it seems important to deeply investigate the biology of CSCs. Much research is focused on targeting essential genes or pathways crucial for cancer development with any therapies against targets expressed by tumour-initiating cells. For example, a comparison of the pathways that regulate stem cell homing with those responsible for metastasis may prove useful too. Treatment of mice with the Hedgehog pathway inhibitor such as cyclopamine inhibits the growth of medulloblastomas in mouse models, without any apparent toxicity (Berman et al., 2002). Thus, the Hg pathway may be inactive in most normal adult tissue, hence minimizing the toxicity effects of these inhibitors (Beachy et al., 2004). Interference with the hedgehog-GLI signalling using a viral vector has been demonstrated to remove resistance to temozolomide in glioblastoma multiforme (Clement et al., 2007). Therefore, GLI regulates stemness and tumour progression and metastatic growth (Altaba et al., 2007). In melanoma, GLI1 function was demonstrated to be striking (Stecca et al., 2007), suggesting that manipulation of GLI1 code could provide a wide-spectrum anticancer target for therapeutic intervention. The finding that oncogenic RAS-AKT pathways regulate GLI1 and that there is a mutual proliferative dependence between oncogenic RAS and HH-GLI function further supports the idea and raises the interesting prospect of using combinatorial therapies targeting oncogenic RAS, AKT and HH-GLI (Stecca et al., 2007). An interesting drug is cyclopamine which is a natural plant alkaloid that is available and effective orally in form and range as well as in laboratory animals with minimal side effects. Cyclopamine treatment has proven good at inhibiting tumour growth and survival of TYr-RASQ61K INK4a–/– melanoma (Stecca et al., 2007). Interestingly, temozolomide has additive and synergistic effects with cyclopamine in glioblastoma stem cell cultures (Clement et al., 2007).

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Lineage Relationships Connecting Germinal Regions to Brain Tumors

Nader Sanai and Arturo Alvarez-Buylla

Abstract Gliomas are a primary cancer of the brain and one of the most lethal cancers known to man. Historically, the neoplastic transformation of fully differentiated glia was widely assumed to be the only mechanism for gliomagenesis. Astrocytes and oligodendrocytes, once thought to be the sole dividing cells in the postnatal brain, were assumed to represent the cellular compartment most susceptible to transformation. More recently, however, this hypothesis has been challenged by the discovery of stem cell and progenitor populations residing in the postnatal brain, which may themselves serve as an origin of brain tumors. Phenotypic and behavioral similarities between gliomas and adult neural stem cells raise the possibility that stem or progenitor cells can give rise to gliomas. Possible candidate cells-of-origin include neuroepithelial cells, radial glia, astrocytic neural stem cells ('B cells'), transient amplifying precursors ('C cells') of the adult subventricular zone (SVZ), or oligodendrocyte progenitor cells of the white matter. While a direct link has yet to be established between any one of these cell types and tumor formation, the different cell lineages arising from the ventricular and subventricular zone during development in the adult may offer clues in deciphering the origin of various tumor subtypes, including gliomas.

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1 Development of Rodent Ventricular and Subventricular Zones

Neural stem cells, defined in vitro as cells that can self-renew and give rise to multiple types of brain cells (Reynolds and Weiss 1992), persist in several regions of the adult rodent brain, but most prominently in the subventricular zone (SVZ), lining the lateral wall of the lateral ventricle. Among these two germinal zones, the SVZ contains the largest reservoir of persistently dividing cells. Progenitors in the SVZ, together with the subgranular zone (SGZ) of the hippocampal dentate gyrus, continue to generate new neurons throughout life (Alvarez-Buylla and Lim 2004).

The development and cellular composition of the rodent SVZ has been described using a combination of cell fate mapping, immunohistochemistry, and electron microscopy (Doetsch et al. 1997; Garcia-Verdugo et al. 1998; Alvarez-Buylla et al. 2001; Merkle et al. 2004; Spassky et al. 2005). Although historically it was believed that neuroepithelial cells in the early neural tube produced two separate pools of committed glial and neuronal progenitors, recent studies support a different model in which neuroepithelial cells either produce or transform into radial glia (Kriegstein et al. 2006; Noctor et al. 2001). Additional data also confirm that neural stem cells are contained within what was classically considered a macroglial lineage (i.e., neuroepithelial cells → radial glia → astrocytes). Thus, soon after birth, radial glial cells within the ventricular zone not only serve as progenitors for many glia (including ependyma; Spassky et al. 2005) and young neurons (both radially and tangentially migrating), but also give rise to the adult SVZ neural stem cells that continue to produce neurons throughout adult life (Alvarez-Buylla et al. 2001).

The cellular composition of the rodent SVZ is organized around slowly dividing astrocyte-like neural stem cells known as type B cells (Doetsch et al. 1999). These cells give rise to actively proliferating type C cells (i.e., transit-amplifying cells), which in turn give rise to immature neuroblasts, called type A cells. In rodents, these neuroblasts are produced by a mosaic of heterogeneous type B cells (Merkle et al. 2007) and migrate to the olfactory bulb via tangentially oriented chains, differentiating into a variety of local interneurons as they reach the olfactory bulb (Doetsch and Alvarez-Buylla 1996; Lois and Alvarez-Buylla 1994). These neuroblast chains are ensheathed by the glial processes of type B cells and, in the anterior and dorsal SVZ, these chains condense to form the rostral migratory stream (RMS; Lois et al. 1996).

More recently, studies have also demonstrated that the SVZ progenitor cells also directly produce oligodendrocytes (Menn et al. 2006). This work suggests that type B cells can generate a subpopulation of transit-amplifying type C cells that begin expressing Olig2. Cells derived from these primary progenitors migrate orthogonal to the orientation of the chains of migrating neuroblasts and invade the white matter to become local oligodendrocyte progenitors (OPCs). These local OPCs act like displaced type C cells and divide locally in the white matter to generate pre-myelinating and myelinating oligodendrocytes.

Therefore, progenitor cells for both neuroblasts and OPCs in the SVZ of the adult rodent brain correspond to type B cells, which have many characteristics of astrocytes. The ventricular surface adjacent to the SVZ is largely covered with multiciliated ependymal cells. Interestingly, recent work has shown that type B cells have a thin apical process between ependymal cells that contacts the ventricle, forming very small apical specializations with a short primary cilium (Doetsch et al. 1997; Mirzadeh et al. 2008). The SVZ also contains blood vessels, microglia, and a substantial extracellular matrix, thought to form the substrate for its unique neurogenic niche (Mercier et al. 2002; Capela and Temple 2006). The architecture of this specialized germinal zone allows for extensive cell–cell interaction and the integration of signals from the ventricular cerebrospinal fluid, the surrounding extracellular matrix, and local blood vessels.

2 Unique Organization of the Human Subventricular Zone

The adult human SVZ also harbors a population of specialized astrocytes and a small subpopulation of this cells proliferates in vivo and functions as adult neural stem cells in vitro (Sanai et al. 2004). These human SVZ astrocytes form a periventricular ribbon that is separated from the lateral ventricular lining by a hypocellular 'gap' region. While there is no evidence of the massive human neuroblast chain migration comparable to that which occurs in rodents or primates (Lois et al. 1996; Kornack and Rakic 2001), a small population of young neurons may still be generated in this region and some of these newborn cells may migrate individually or in small groups to the olfactory bulb. Nevertheless, the magnitude and mechanism of neuronal proliferation and migration in the adult human SVZ, as well as the persistence of human RMS, remains controversial (Sanai et al. 2004, 2007; Curtis et al. 2007). Similarly, the suggestion that an open olfactory ventricle, linking the anterior horn of the lateral ventricle to the olfactory bulb, is not supported by previous work (Sanai et al. 2004; Bedard and Parent 2004; Humphrey 1940; Mueller et al. 2005; Muller and O'Rahilly 1989; Nakashima et al. 1985).

Recently, the cellular composition and cytoarchitecture of the adult human SVZ has been characterized in detail (Quinones-Hinojosa et al. 2006),

demonstrating a variable thickness in its layering and cell density, depending upon the region along the anterior–posterior extent of the lateral ventricular system. In general, four layers to the adult human SVZ can be identified: the inner most layer, a monolayer of ependymal cells lining the ventricular cavity (layer I); a hypocellular gap (layer II) abutting the ependymal layer; a ribbon of astrocytes, some of which may function as neural stem cells (layer III); and a transitional zone (layer IV) into the overlying brain parenchyma. Using both immunohistochemical and ultrastructural analyses, oligodendrocytes have also been observed in the astrocyte ribbon of layer III, a layer which only exists along the lateral ventricles and not along the third ventricle, fourth ventricle, or the medial (septal) wall in humans.

3 Glial Progenitors in the Subcortical White Matter

Beyond the ventricular and subventricular zones, a loosely organized population of glial progenitors accounts for 2% of the adult rat white matter and as many as 4% of the adult human subcortical white matter (Assanah et al. 2006; Nunes et al. 2003). Although these cells are typically non-migratory and slowly proliferating in vivo (Roy et al. 1999; Noble et al. 1992), in vitro studies have shown that adult glial progenitors can be induced to become more migratory and proliferative when treated with growth factors including PDGF, EGF, FGF, and glial growth factor (Wolswijk et al. 1991; Wolswijk and Noble 1992; Shih and Holland 2006). Using flow-cytometry techniques, these cells can also be extracted and identified on the basis of their expression of an immature neural ganglioside recognized by monoclonal antibody A2B5 (Nunes et al. 2003). They do not, however, express more mature glial markers and in vitro can be induced to function as neural stem cells in the presence of exogenous growth factors. Subcortical white matter glial progenitors serve an unknown function in the adult human brain, but represent a large source of parenchymal progenitors that could be involved in gliomagenesis, or even serve as a possible cell-of-origin for some gliomas. Unfortunately, very little is known about the lineage relationships of these cells, their putative microenvironment, or their relationship to the subventricular zone.

4 Emergence of the Cancer Stem Cell Hypothesis

Germinal regions such as the subventricular zone have long been suspected as a possible source of gliomas (Vick et al. 1977; Lantos and Cox 1976). Not surprisingly, many gliomas are either periventricular or contiguous with the ventricular or subventricular zone (Lim et al. 2007) and contain cells possessing phenotypic and behavioral characteristics shared by neural stem cells. Features common to both progenitor cells and gliomas include high motility, potential to

produce several types of progeny, robust proliferative potential, and association with blood vessels and white matter tracts. CD133 (also known as prominin) is a cell surface marker associated with stem-like cells found in both the hematopoietic and central nervous system (Singh et al. 2003). Interestingly, CD133 is also expressed by radial glia and neuroepithelial cells (Pfenninger et al. 2007). On the basis of CD133 expression, cancer stem cells and normal adult neural stem cells can be isolated by cells sorting and shown to self-renew and exhibit multipotency (Singh et al. 2004a; Galli et al. 2004). The identification of this population suggests that these cells represent the tumor-initiating fraction of human gliomas (Singh et al. 2004b) and has led to the emergence of the 'cancer stem cell hypothesis' for brain tumors.

5 Susceptibility of Stem and Progenitor Cells to Transformation

That neural stem cells are potentially susceptible to transformation is suggested by the fact that, in animal models, regions of the brain that are highly proliferative—including areas with neural stem cell populations—are more sensitive to chemical or viral oncogenesis than are areas with a low proportion of proliferating cells. In canine and rodent brains, for example, avian sarcoma viral transformation (Hopewell 1975) or systemic exposure to the carcinogen *N*-ethyl-*N*-nitrosourea (ENU; Pilkington and Lantos 1979; Lantos and Pilkington 1979; Lantos 1977) preferentially leads to tumor formation in the proliferative subventricular zone rather than in nonproliferative regions of the brain. In one study, intraventricular inoculation with avian sarcoma virus in neonatal canine brains initially led to periventricular glioma microfoci, but as the tumors grew, their continuity with the subventricular zone diminished until, at day 10 after inoculation, they were found in the deep white matter without apparent connection to the subventricular zone (Pilkington and Lantos 1979). Migration of transformed germinal zone cells may be a mechanism by which human gliomas arise from neural stem cells but then go on to lose any evidence of continuity with these regions. Similarly, infusion of EGF or PDGF into the lateral ventricle of the adult brain results in the elevated proliferation of progenitor cells and the formation of highly invasive cells or glioma-like masses (Jackson et al. 2006; Doetsch et al. 2002a). Mouse models in which progenitor cells are exposed to high levels of PDGF or EGF exhibit the formation of tumors that are histologically similar to human gliomas, and alterations in the PDGF and EGF pathways have frequently been found in human glioma (Feldkamp et al. 1997). Finally, mice lacking the tumor suppressors p53 and NF1 in the CNS develop early lesions that are associated with the SVZ and that progress to tumors resembling human malignant astrocytomas (Zhu et al. 2005).

As the postnatal brain ages, the germinal region neural progenitor proliferation appears to be restrained by increased levels of cell-cycle inhibitor proteins.

Although cell-cycle inhibition may act to prevent hyperproliferation and tumor development as oncogenic mutations accumulate in an aging organism, this inhibition also limits the ability of the organism to maintain normal neural tissue homeostasis. In aging mice, the cell-cycle inhibitor p16/INK4A is expressed in the SVZ, but this expression is absent in younger mice (Campisi 2007). When p16/INK4A is removed, SVZ cells from aging mice seem to retain the proliferative potential observed in younger mice (Molofsky et al. 2006). p16/INK4A is frequently lost or mutated in human gliomas (Ivanchuk et al. 2001), suggesting that disruption of the regulation of proliferative potential, when coupled with other oncogenic events, could result in cancer formation. Importantly, while it has been speculated that brain tumor stem cells arise from SVZ stem and/or progenitor cells that have sustained oncogenic mutations, no direct evidence links adult neural stem cells to a glioma cell-of-origin.

The subcortical white matter contains one of the largest populations of cycling cells in the adult brain (Roy et al. 1999). Progenitor cells in the white matter are another cellular compartment that may be uniquely susceptible to transformation. High levels of PDGF expression in the subcortical white matter of rodents can lead to formation of heterogeneous glioma-like masses that are driven by a mixture of PDGF expression, tumor-initiating cells, and recruited local progenitors (Assanah et al. 2006). Interestingly, the pattern of infiltration seen in this model demonstrates a predilection for fiber tracts, as seen in both human gliomas and glial progenitors during brain development (Kakita and Goldman 1999; Kakita et al. 2003), raising the possibility that the infiltrative capacity of gliomas may represent a re-expression of a neonatal glial progenitor phenotype.

6 Shared Immature Expression Profiles Among Brain Tumors and Stem Cell Niches

Postnatal germinal regions such as the ventricular and subventricular zones contain unique cytoskeletal proteins, tumor suppressor genes, growth factors, transcriptional signaling cascades, and telomerase expression patterns that are shared by glioma cell populations. These unique phenotypes, along with tumor location and age of onset, suggest possible lineage relationships associating normal germinal zone progenitor cell types with specific histologic subtypes of brain tumors.

6.1 Cytoskeletal Proteins

Nestin is an intermediate filament widely expressed by progenitor and stem cells during development (Wiese et al. 2004). Although it is relatively prevalent at birth, its expression is downregulated in the adult brain and becomes restricted

to the SVZ postnatally. Interestingly, following ENU exposure, undifferentiated Nestin+ cells are present during the earliest periods of periventricular tumorigenesis and these cells persist throughout tumor progression (Jang et al. 2004). Similarly, Nestin+ SVZ cells removed at birth can undergo spontaneous immortalization after in utero exposure to ENU (Savarese et al. 2005). Nestin+ cells also exist in human gliomas (Yang et al. 2008), further supporting the hypothesis that neural stem cells may be implicated in glioma formation. It remains unclear, however, whether the expression of Nestin is a genetic aberration or a normal property of the precursor cell from which the tumor originated. Furthermore, since Nestin is nearly ubiquitously expressed by neural stem and progenitor cells at all stages of development, its utility as a stem cell or brain tumor stem cell marker is limited.

Doublecortin is a microtubule-associated protein that is expressed by the SVZ type A cells, as well as by migrating young neurons generated by neuroepithelial cells and radial glia (Nacher et al. 2001). While many of these cells are postmitotic, type A cells in the SVZ function as neuroblasts and divide to generate additional neuronal progeny (Luskin 1998). Doublecortin has also been shown to be preferentially expressed in high-grade gliomas (Daou et al. 2005) and glioneuronal tumors such as gangliogliomas (Becker et al. 2002). Interestingly, overexpression of doublecortin in glioma cell lines appears to protect tumor cells from severe oxygen and glucose deprivation (Santra et al. 2006). Taken together, these findings raise the possibility that neuronal precursors, possibly similar to type A cells, may be implicated not only in gliomagenesis, but also in the formation of glioneuronal tumors, many of which develop early in life.

6.2 *Tumor Suppressor Genes*

p53 is a tumor suppressor gene expressed in SVZ cells (Gil-Perotin et al. 2006) and frequently deleted or mutated in gliomas (Sidransky et al. 1992). Loss of p53, alone, is not sufficient for tumor formation, but may predispose SVZ cells to tumorigenesis. Specifically, it increases the number of adult neural stem cells and neuroblasts and induces the formation of periventricular hyperplasia in adult SVZ, while also impairing differentiation (Gil-Perotin et al. 2006). Thus, loss of p53 confers a proliferative advantage to the slow- and fast-proliferating populations in adult SVZ. Synergistically, prenatal exposure of $p53^{-/-}$mice to ENU leads to the formation of glioma-like tumors in the adult SVZ (Katayama et al. 2005). Here, tumor formation has been suggested as the result of amplified self-renewal, faster cell division of the relatively quiescent population, and impaired differentiation of multipotential progenitors. Based upon this model, transformation of the adult SVZ is preceded by recruitment of the quiescent self-renewing (type B cell) population to the fast-proliferating (type C cell) compartment, which in turn may be unable to differentiate along distinct

lineages. Several other signaling pathways also shape this response, as p53$^{-/-}$ mice form gliomas following both PDGF overexpression (Hesselager et al. 2003) and Ras activation (Zhu et al. 2005). Interestingly, among non-neurogenic astrocytes, loss of p53 combined with constitutive EGF-receptor (EGFR) activation does not lead to tumorigenesis, even though these cells have the ability to generate gliomas when expression of constitutively active EGFR is associated with loss of other cell-cycle genes, such as *Ink4a* and *Arf*. Of note, 60% of malignant human gliomas show abnormalities at the *Ink4a/Arf* locus and *Ink4a/Arf* mutations have also been detected in cells isolated from ENU-injected animals (Savarese et al. 2005). These observations therefore identify at least two distinct mechanisms of gliomagenesis: one that is *Ink4a/Arf* dependent and p53-independent and another that is p53-dependent. This is consistent with human glioma studies suggesting that p53 and *Ink4a/Arf* mutations are mutually exclusive (Bachoo et al. 2002).

Early inactivation of p53 has also been shown to cooperate with the neurofibromatosis-1 (NF1) tumor suppressor gene mutation, inducing malignant astrocytoma formation in a mouse tumor model (Zhu et al. 2005). The NF1 tumor suppressor product neurofibromin is a functional Ras GTPase-activating protein and its loss results in abnormal activation of Ras, a central mediator of receptor tyrosine kinase (RTK) signaling. Mice that carry germline mutations in both p53 and NF1 developed both low- and intermediate-grade astrocytomas. These astrocytomas express Nestin, the progenitor-associated intermediate filament, and were associated with the SVZ, suggesting that SVZ cells are most susceptible to p53/NF1-mediated astrocytoma formation.

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a recognized tumor suppressor mutated in many gliomas. The PTEN protein is a phosphatidylinositol phosphate (PIP) phosphatase that lowers PIP3 levels and enhances the rate of apoptosis. PTEN also decreases cell motility via G protein-coupled mechanisms. PTEN is expressed by neuroblasts during neuronal differentiation (Lachyankar et al. 2000). Using a knockout mouse model, PTEN has also been shown to regulate type A cellular migration from the SVZ to the olfactory bulb (Li et al. 2003). Importantly, loss of PTEN increases proliferation in the SVZ and decreases apoptosis, indicating that PTEN is a negative regulator of progenitor cell proliferation. As such, PTEN is a common pathway shared by both SVZ type A cells and secondary glioblastomas.

6.3 Growth Factors

Growth factor signaling pathways play a critical role in both gliomagenesis and germinal zone regulation. For example, nearly half of all high-grade astrocytomas demonstrate EGFR amplification, while EGFR mutation is a classic step in the development of primary glioblastomas. EGF-responsive C cells and a

subpopulation of B cells within the adult SVZ constitute a large population of dividing progenitors in the adult brain. EGF-mediated stimulation of this population prevents C cell differentiation and induces their migration, leading to an infiltrative phenotype comparable to that seen among high-grade gliomas (Doetsch et al. 2002a).

A population of platelet-derived growth factor (PDGF) + type B cells have also been identified in the adult rodent SVZ. These cells can give rise to both neurons and oligodendrocytes in vivo (Jackson et al. 2006). With excessive PDGF activation, the rodent SVZ arrests neuroblast production, induces SVZ cellular proliferation, and creates areas of hyperplasia with features of early glioma formation (Jackson et al. 2006). This behavior change represents a potential link between these PDGFR + type B cells and the early changes associated with tumor initiation. Considering the frequency of PDGFR over-expression among both low- and high-grade gliomas, these findings raise the possibility that transformed SVZ type B cells could serve as a source of gliomas and primary glioblastomas.

6.4 Vascular Niches

A strong association exists between vascular and neurogenic niches in adult germinal regions (Gilbertson and Rich 2007; Palmer et al. 2000; Tavazoie et al. 2008). Gliomas are typically hyper-vascularized and associated with neovascular beds (Fischer et al. 2005). Not only are stem and progenitor cells typically clustered around or in contact with blood vessels (Palmer et al. 2000), but vascular endothelial growth factor (VEGF) and its receptors are expressed by neurospheres derived from rodent SVZ (Knizetova et al. 2008). VEGF has also been implicated in glioma growth and it is secreted by glioma cells that act on tumor endothelial cells expressing VEGF receptors (Jain et al. 2007). Similarly, cancer stem cells isolated from gliomas also generate markedly elevated levels of VEGF (Bao et al. 2006a). Interestingly, type B cells in the rodent are known to extend basal processes that specifically contact blood vessel endothelia (Mirzadeh et al. 2008). Taken together, these data suggest that similar microenvironments promote both stem/progenitor cell growth and tumorigenesis and also that targeting proangiogenic factors may be a therapeutic strategy against the putative cancer stem cell fraction.

6.5 Transcription Factors

If neural stem cells are the source of tumor initiation, their progression toward a tumorigenic state may be achieved through the operation of abnormal developmental programs (Wechsler-Reya and Scott 2001). Multiple developmental signaling pathways associated with normal stem/progenitor cell function may

serve as critical regulators of tumorigenesis. Although these programs coordinate the use of normal cellular components, their timing, order, and magnitude are probably abnormal in tumorigenic states.

Sonic Hedgehog (Shh) is a key regulator of progenitor proliferation in the external granular layer (EGL) of the cerebellum (Wechsler-Reya and Scott 1999; Mullor et al. 2002). Misregulation of this pathway by mutation of the Shh receptor, Patched, or constitutive activation of Smoothened results in medulloblastoma formation, linking them with cerebellar granule cell precursors (Kenney et al. 2003; Kim et al. 2003; Rubin and Rowitch 2002). Furthermore, granule cell precursors of the EGL can be considered a type of transit-amplifying cell (C cell), derived from primary progenitors of the rhombic lip. Therefore, the direct effect of Shh misregulation in medulloblastoma is a good example of the possible role of transit-amplifying cells as a cell-of-origin in human brain tumors.

Hedgehog signaling is also implicated in both glioma and progenitor proliferation, as it activates two zinc-finger transcription factors (Gli1 and Gli2) and suppresses Gli3 function, which, in turn, regulate progenitor cells by promoting cell-cycle entry and DNA replication. In the adult CNS, Gli1 is expressed by neuronal progenitors in the SVZ, where the sonic hedgehog (Shh)–Gli pathways maintain the type B cell population and facilitate the survival and proliferation of their progeny (Palma et al. 2005). Importantly, Gli1 is expressed in both low-grade and high-grade gliomas, and the Shh–Gli pathway may mediate the initiation and maintenance of these tumors as it does for neural stem cells (Dahmane et al. 2001). Therefore, as would be expected, treatment with cyclopamine, a hedgehog pathway inhibitor, represents a potential avenue of glioma stem cell control (Bar et al. 2007).

7 Transit-Amplifying Type C Cells as a Glioma Cell-of-Origin

Although it remains possible that neuroepithelial cells, radial glia, type B cells, type A cells, and even a putative astrocyte precursor cell (APC) could each serve as a glioma cell-of-origin (see Fig. 1), it seems likely that a type C cell would be most commonly implicated in a fashion similar to the transit-amplifying cells of the EGL. As mentioned above, the EGF signaling pathway plays an important part in both gliomagenesis and adult neural stem cell regulation. Amplification of the EGFR gene is associated with the formation of high-grade gliomas (Kuan et al. 2001; Lassman 2004), and activation of EGFR promotes the growth of both astrocyte precursors and neural stem cells (Doetsch et al. 2002a). As many as 50% of high-grade astrocytomas demonstrate EGFR amplification, and EGFR activation may drive the transformation process in the development of gliomas. In the SVZ of the adult rodent, the majority of EGF-responsive cells correspond to the rapidly dividing transit-amplifying C cells. In response to EGF exposure in vitro, these cells give rise to spherical

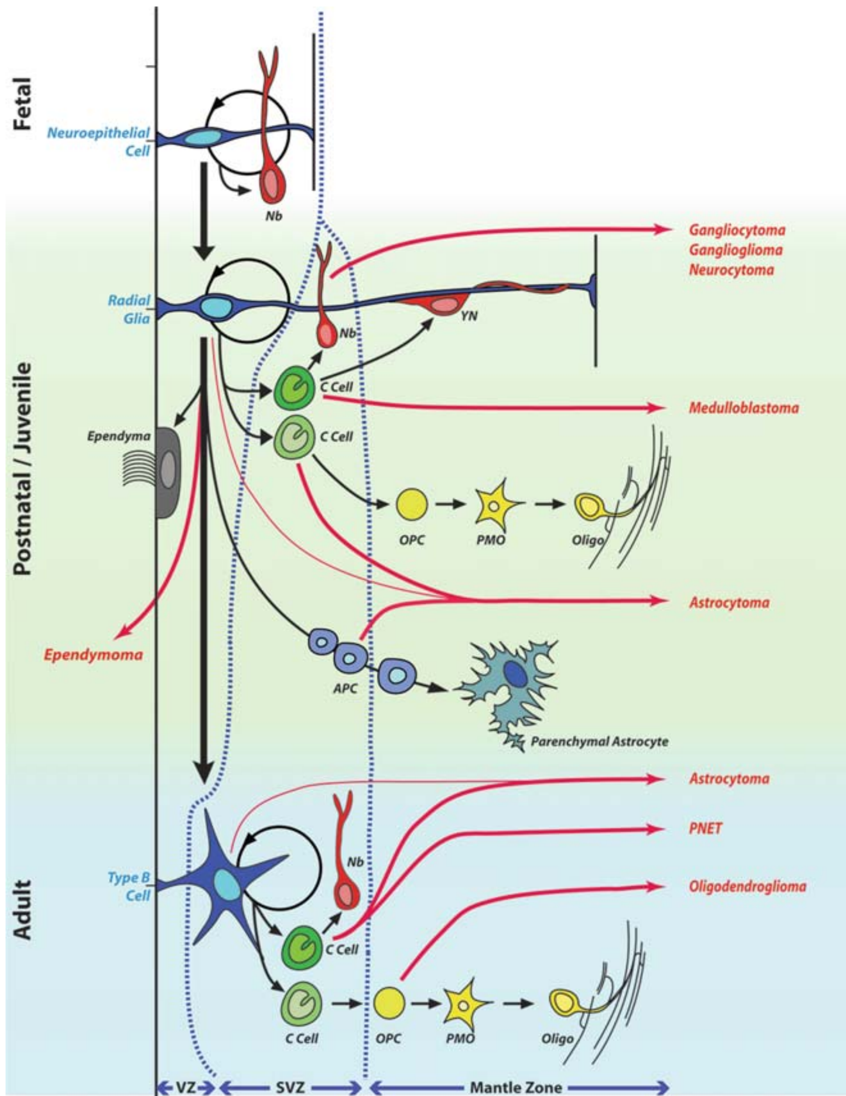

Fig. 1 New understanding of the stem cell lineage (neuroepithelial cells → radial glia → type B cells) and its derived progenitors suggest possible cells-of-origin for different types of brain tumors. During embryonic development, ventricular zone neuroepithelial cells and radial glia function as the primary precursors of young neurons (*YN*) and glial cells. During the perinatal period, a subpopulation of periventricular radial glia assumes this function as the primary progenitors. These radial glia have been suggested as a cell-of-origin for ependymomas. They produce young neurons and oligodendrocytes through intermediary progenitors (type C cells), as well through oligodendrocyte precursor cells (*OPCs*) and premyelinating oligodendrocytes (*PMOs*). Collectively, these transit-amplifying cells are strong candidates as cells-of-origin for multiple types of tumors. Parenchymal astrocytes are also produced by radial glia, possibly through one or more astrocyte precursor cells (*APCs*) that may also divide. Thus,

collections of cells (neurospheres) that are multipotential and self-renewing. Interestingly, type C cells exposed to exogenous EGF for 6 days in vivo demonstrate unusually high motility, migration along blood vessels and white matter tracts, and expression of EGFR and tenascin—all hallmark characteristics of gliomas. Furthermore, type C cell proliferation is tightly regulated by the CDK inhibitor p27Kip1, a G_1 regulator of the cell cycle. Elimination of this regulator selectively increases the number of type C cells, concomitant with a decrease in the number of type A neuroblasts (Doetsch et al. 2002b). Interestingly, a low expression level and high degradation activity of p27 are independent poor prognostic factors in patients with malignant gliomas (Zolota et al. 2008). Taken together, these data suggest that not only could type C cells be a driving force behind neurosphere formation, but that the response of type C cells to exogenous growth factors may serve as a model system to study tumor initiation and invasion in the adult brain. Importantly, the human homologue to the C cell type has yet to be identified.

8 Implications for Brain Tumor Therapy

Based upon the cancer stem cell hypothesis, any brain tumor therapy that fails to eradicate cancer stem cells will result in the recurrence or regrowth of the remaining tumor stem cells, leading to eventual disease progression (Schulenburg et al. 2006). As this cell population is characterized in more detail, studies have suggested that brain tumor stem cells are particularly resistant to the standard adjuvant therapies for gliomas, including radiation therapy (Bao et al. 2006b) and the alkylating agent, temozolomide (Clement et al. 2007). Thus, new therapies that target the most resilient fraction of the tumor are needed to improve treatment response.

One such strategy relies upon our understanding of mechanisms governing the fate of adult neural stem cell progeny, where factors that inhibit proliferation of neural stem and progenitor cells could be directed toward brain tumor stem cells. Bone morphogenetic proteins (BMPs) are a family of cytokines with a complex set of effects on neural stem and progenitor cells (Varga and Wrana

Fig. 1 (continued) radial glia, as well as C cells and APCs, may represent a source of gliomas. In the external granule layer (EGL) of the developing cerebellum, transit-amplifying cells similar to type C cells have also been suggested as a source of medulloblastomas. Migrating neuroblasts (*Nb*) represent a population comparable to OPCs, but along a neuronal lineage, and thus may generate tumors with neuronal components, including gangliocytomas, gangliogliomas, and neurocytomas. In the subventricular zone, astrocytic neural stem cells (type B cells) may themselves be a source of gliomas, as might their C cell progeny, which also could be a source of primitive neuroectodermal tumors (PNETs) and gliomas. These proposed lineage relationships connecting the ventricular and subventricular zones to brain tumors remain hypothetical and as of yet unproven

2005). In neural stem cells derived from early embryos, BMPs appear to promote both proliferation and neuronal differentiation. However, these stem-like tumor cells may be susceptible to stimuli that alter the proliferation of normal neural precursors. Treatment of this tumor stem cell population with BMPs, which limit the proliferation of normal stem cells, blocks the ability of these cells to form tumors upon transplantation. In contrast, neural stem cells derived from older animals undergo astrocytic differentiation in response to BMPs (Panchision et al. 2001). Preclinical trials are underway to assess the utility of such an agent in curtailing glioma proliferation (Nakano et al. 2008).

Other studies have focused upon the migratory abilities of specific neural stem cell lines, which may have the potential to deliver therapeutic substances to specific sites in the brain (Aboody et al. 2000). Neural stem cells transplanted into animal models of brain neoplasia were found near metastatic tumor cells far from the site of their transplantation (Tang et al. 2003). These observations suggest that neural stem cells engineered to deliver cell-specific cytotoxic agents might be used to track down and destroy malignant cells. This would be particularly useful if non-cycling cells could be targeted, as they often represent the tumor fraction that survives adjuvant chemo- and radiation therapy regimens (Hambardzumyan et al. 2006).

9 Conclusions

A new path has emerged in the clinical neurosciences, connecting neuro-oncology with developmental neurobiology. The juvenile and adult human brain, for many years thought to be composed of fully differentiated cells contains multiple neuronal and glial progenitor cell populations, most notably in the subventricular zone and white matter. As we elucidate the lineage relationships of the ventricular and subventricular zone, from embryonic stages to adulthood, it may eventually be possible to identify stem or progenitor cell types at risk for transformation. This will allow the design of mouse tumor models in which specific populations of progenitor cell are targeted. A revised pathological classification system of tumors may emerge that will link tumor subtypes to specific developmental lineages. This, in turn, could facilitate the discovery of new markers for tumor progression, techniques for earlier cancer detection, and novel targeted therapies. Our success, however, depends largely upon our ability to understand the natural biology of these poorly understood germinal regions.

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