Enrique Grande · Luis Antón-Aparicio
Editors

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Foreword

This textbook is about the development of a novel area of molecular biology: stem cells in cancer.

The term "cancer" describes a cluster of diseases characterized by uncontrolled cellular growth, cellular invasion into adjacent tissues, and the potential to metastasize if not treated at a sufficiently early stage. These cellular aberrations arise from accumulated genetic modifications, either via changes in the underlying genetic sequence or from epigenetic alterations (e.g., modifications to gene activation- or DNA-related proteins that do not affect the genetic sequence itself). Tumors and other structures that result from aberrant cell growth contain heterogeneous cell populations with diverse biological characteristics and potentials. As such, a researcher sequencing all of the genes from tumor specimens of two individuals diagnosed with the same type of lung cancer will identify some consistencies along with many differences.

The traditional view among oncologists is that cancerous tumors are the result of genetic mutations within ordinary cells that cause them to divide uncontrollably and then spread. However, evidence has been mounting that small numbers of stem cells within tumors actually orchestrate their growth and proliferation.

A consensus panel convened by the American Association of Cancer Research has defined a CSC as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor." Stem cells are essential to maintain regenerative tissues and are a critical component of repair processes in response to tissue injury and infection.

The cancer stem cell (CSC) hypothesis has been the focus of hundreds, if not thousands, of journal articles over the past decade. Indeed, it has been one of the most hotly debated questions in cancer research: do tumors return despite powerful treatments because they harbor a small number of stem cells that have the capabilities to evade drugs? If these rare, resilient cells are seeding new tumors, it would suggest a radical change in strategy for fighting cancer: drugs should primarily target these rare stem cells, rather than aiming to shrink a tumor. But research has produced conflicting results about the existence and importance of such cancer stem cells.

In 1994, Lapidot and colleagues provided the first solid evidence to support the CSC hypothesis when they used cell-surface protein markers to identify a relatively rare population of stemlike cells in acute myeloid leukemia (AML). Actually, the concept dates to the mid-nineteenth century. In 1855, German pathologist Rudolf Virchow proposed that cancers arise from the activation of dormant, embryonic-like cells present in mature tissue.

More recently, CSCs have been also isolated from solid tumors. Characteristics of these tumor-initiating cells have been thoroughly discussed and CSCs have been defined by tumorigenicity in immunocompromised mice and, more importantly, the ability to generate heterogeneous cancer cell populations within the resulting tumors that are phenotypically similar to the original tumor.

Questions concerning the close relationship between CSCs and cancer still need further research in order to get a clear answer: How do cancer stem cells arise? The molecular pathways that maintain "stem-ness" in stem cells are also active in numerous cancers. According to different open research lines, cancer stem cells may arise from mutated stem cells; also it has been proposed that the origin of CSCs may be a progenitor cell that undergoes two or more mutations, and finally it has been proposed that a fully differentiated cell may undergo several mutations that drive it back to a stemlike state.

The discovery of CSCs in some tumor types has ushered in a new era of cancer research. Cancer stem cell science is an emerging field that will ultimately impact researchers' understanding of cancer processes. It will help to a better understanding of the so-called plague of the twenty-first century and may help biomedical research to identify new therapeutic strategies.

The question "Are stem cells involved in cancer?" may not have a simple answer. This book tries to gather the ongoing investigations about this topic. Each topic is presented and discussed in order to give to the reader a broad spectrum of information.

The authors aim to help the reader to make up his own mind about this important issue.

Madrid, Spain Jesus Garcia-Foncillas Lopez
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Absence of evidence is not evidence of absence

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Abstract In 1978, Schofield hypothesized the existence of cells in the proximity of stem cells, termed the stem cell niche, that have the ability to extrinsically exert influence on stem cell behavior. Indeed, a large body of evidence from a number of stem cell systems validated this hypothesis by affirming the critical importance of stem cell niche interactions and localized extracellular signals in regulating stem cell self-renewal and differentiation. This concept is precisely illustrated in the bone marrow transplantation setting in which the success of the transplant is contingent on the ability of hematopoietic stem cells (HSCs) to home and seed appropriate secondary supportive niches of intravenous infection.

Experiments using parabiosis of genetically marked strains of mice demonstrated that HSCs constitutively migrate through blood and are able to re-engraft unconditioned bone marrow to niche function HSCs have emerged as the model system to study tissue-specific niche stem cells and their potential to regenerating secondary niche.

This chapter reviews observations, olds and recents, suggesting that this plasticity may perhaps outstrech the marrow boundaries, so that HSCs (mesodermal in origin) can give rise to cells that normally derive from germ layers other than the mesoderm.

This review discusses the inextricable relationship between adult stem cells and bone marrow-derived hematopoietic cells, and their roles in replenish adult stem cell niches.

Keywords Cancer • Mesodermal • Stem cells • Stemness • Tumor niche

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1.1 Introduction

Among stem cells, adult stem cells are often localized into specific niches where they utilize many, but not necessarily all, of the external and intrinsic factors used by the embryonic counterparts in selecting a specific fate. Within the niche, stem cells are able to maintain their ability for self-renewal as well as their potential so that, consequently, detachment from the niche compartment induces stem cell differentiation and loss of self-renewal. Thus, when a stem cell begins to divide, it is thought that one daughter cell remains into niche to replace the original stem cell whereas other daughter cell is expelled out of niche and starts its process of differentiation. In this process, a cell retains self-renewal and differentiation inhibitory factors, so that keep being stem cell, whereas another daughter cell is destined to proliferate during a certain number of divisions for finally differentiate along a particular lineage. This latter daughter cell will receive too few stemness factors to maintain as stem cell, and/or inherit proliferation and/or differentiation factors that can overcome its stem cell phenotype. To maintain tissue homeostasis and correct functioning of organism, the number of daughter cells that retain stem cell identity must be strictly controlled such that differentiated cells can be generated in response to any injury. Likewise, the rate of division of stem cells into niche must be tightly controlled since an overproduction of daughter cells destined to be differentiated may be harmful because may result in cancer generation. In the present chapter, we speculate cancer stem cell niche for as well as the mechanisms that influence on the generation of daughter cells.

Bone marrow has received increasing attention during the last several years from researchers hoping to reveal a universal stem cell. There is two strongly reasons: bone marrow has abilities for pluripotent differentiation and for organs system in which a native stem-cell region has not yet been identified, such as the kidney. In this case bone marrow may serve as an source alternative. The bone marrow has multiple types of stem cells, and numerous studies have reported differentiation when these cells engraft in other organ systems.

The discovery that adult hematopoietic stem cells can cross lineage boundaries to become cells of other tissues has challenged the traditional view that somatic stem cells are lineage-restricted and organ-specific.

One possibility is that some hematopoietic stem cell retain developmental plasticity and can be reprogrammed to express genes that are required to differentiate into the cells of the organs into which they are introduced. Using bioinformatics and microarrays analysis, there are observed that these primitive cells exhibit distinct gene expression patterns compared with less primitive bone marrow cells, suggesting that differentially expressed genes may guide the phenotype and function of hematopoietic cells.

1.2 Concept of Stem Cells

Stem cells are defined as cells that have clonogenic and self-renewing capabilities and that differentiate into multiple cell lineages. More explicitly, stem cells can generate daughter cells identical to their mother (self-renewal) as well as produce

progeny with more restricted potential (differentiated cells) [1]. Whereas embryonic stem cells are derived from mammalian embryos in the blastocyst stage and have the ability to generate any terminally differentiated cell in the body, adult stem cells are part of tissue-specific cells of the postnatal organism into which they are committed to differentiate [2].

Stem cells are not only units of biological organization, responsible for the development and the regeneration of tissue and organ systems, but also are units in evolution by natural selection. Stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage differentiation. Stem cells can be divided into a long-term subset, capable of indefinite self-renewal, as well as a short-term subset that self-renews for a defined interval. Stem cells give rise to non–self renewing oligolineage progenitors, which in turn give rise to progeny that are more restricted in their differentiating potential, and finally to functionally mature cells. The earliest stem cells in ontogeny are totipotent, extending from the zygote to the inner cell mass of the blastocyst; soon thereafter, totipotent stem cells give rise to somatic stem/progenitor cells and primitive germline stem cells [3].

A number of properties besides self-renewal and differentiation potential are frequently ascribed to stem cells, including the ability to undergo asymmetric cell divisions, exhibit extensive self-renewal capacity, exist in a mitotically quiescent form, and clonally regenerate all of the different cell types that constitute the tissue in which they exist [4].

There are three defining features of a stem cell on which all can agree [5]:

- A stem cell "self-renews" – that is, when a stem cell is called into action, it undergoes cell division. One daughter cell remains a stem cell, while the other becomes more committed to forming a particular cell type (a "committed progenitor") by a process called "asymmetric division".
- A stem cell forms multiple cell types (that is, it is "multipotent").
- A single stem cell completely re-forms a particular tissue when it is transplanted within the body.

On the basis of these three defining features, several others are implied, but are not necessarily true of all stem cells [5]:

- *Self-renewal = extensive proliferation:* The ability to self-renew has been linked conceptually to a stem cell's ability to divide extensively to form vast numbers of cells. However, a stem cell is not immortal, but is endowed with a certain restricted capacity to self-renew related to how fast a tissue turns over.
- *Clonogenicity = stemness:* A stem cell is thought to be "clonogenic entities", which means that it can proliferate to form a colony of cells. However, while clonogenicity is part of the essential assay in defining a stem cell (that is, a single cell capable of proliferating and forming multiple cell types), not all cells that form colonies qualify as stem cells.
- *Stemness = undifferentiation:* In many cases, a stem cell is thought to be an undifferentiated cell type (that is, it does not have a mature phenotype), but there are instances in which a cell with differentiated character can behave as a stem cell.

1.2.1 Stem Cell Types

Although all stem cells share the three characteristics listed above, they are not necessarily equal in their ability to form multiple cell types, and a hierarchy exists:

- *totipotent:* the fertilized egg, capable of independently giving rise to all embryonic and extra embryonic tissues;
- *pluripotent:* the inner cell mass of the blastocyst in the developing zygote and embryonic stem cells in culture, capable of giving rise to all embryonic cells and tissues;
- *multipotent fetal stem cells:* cells derived from the three embryonic germ layers (ectoderm, mesoderm and endoderm) that become more and more committed to generating particular cells as organs and tissues are formed.
- *multipotent adult stem cells:* thought to be tissue-specific and sometimes form only one type of cell (unipotent).

In conclusion, a working definition of a stem cell is a clonal, self-renewing entity that is multipotent and thus can generate several differentiated cell types. Admittedly, this definition is not applicable in all instances and is best used as a guide to help describe cellular attributes [1].

Stem-cell populations are nowadays shown to be in specific anatomical locations, termed niches, that regulate how they participate in tissue generation, maintenance and repair.

1.2.2 Origin of Stem Cells

The origin or lineage of stem cells is well understood for ES cells; their origin in adults is less clear and in some cases controversial. The paucity of information on the developmental origins of adult stem cells leaves open the possibility that they too escape lineage restriction in the early embryo and subsequently colonize specialized niches, which function to both maintain their potency as well as restrict their lineage potential. Alternatively, the more widely believed, though still unsubstantiated, model for the origin of adult stem cells assumes that they are derived after somatic lineage specification, whereupon multipotent stem cells-progenitors arise and colonize their respective cellular niches [1].

1.2.2.1 Embryonic Stem Cells

The development of mouse ES cells in 1981 [6, 7] provided the paradigm and much of the technology for the development of human ES cells [8].

The gold standard of stem cells is the fertilized egg, which produces an organism replete with a myriad of specialized cell types, including reproductive germ stem cells

(GSCs). As the embryo first develops, an outer protective shell of support cells encases an undifferentiated mass of pluripotent embryonic stem cells (ESCs) that will make the animal. As development proceeds, pluripotent embryonic stem cells disappear as more restricted somatic stem cells (SSCs) give rise to the tissues and organs [9].

Mouse and human ES cells are derived directly from the inner cell mass of pre-implantation embryos after the formation of a cystic blastocyst. This population of cells would normally produce the epiblast and eventually all adult tissues, which may help to explain the developmental plasticity exhibited by ES cells [1].

At the egg cylinder stage of embryonic development, a population of cells near the epiblast can be identified as primordial germ cells (PGCs). These PGCs migrate to and colonize the genital ridges, where they produce mature germ cells and generate functional adult gametes [1]. Undifferentiated PGCs are germline stem cells because significant numbers of PGCs are derived from smaller numbers of precursors (self-renewal), PGC progeny potentially include either oogonial or spermatogonial cells (multilineage differentiation), and PGCs cultured in vitro can retain multipotentiality [3].

Embryonic germ (EG) cells have many of the characteristics of ES cells with respect to their differentiation potential and their contribution to the germ line of chimeric mice. The most notable difference between ES and EG cells is that the latter may display considerable imprinting of specific genes [1]. In considering the properties of ES or EG cells, there are certain generic features that any ES cell might be expected to possess, and other properties which may be peculiar to bona fide pluripotent cells isolated from different species or different tissues or representative of a different stage of embryonic development. The mouse ES cell provides a benchmark for definition of the generic requirements for ES cells. Its key features are these: it is derived from a pluripotent cell population; it is stably diploid and karyotypically normal in vitro; it can be propagated indefinitely in the primitive embryonic stage; it can differentiate spontaneously into multiple cell types representative of all three embryonic germ layers; and it can give rise to any cell type in the body, including germ cells, when allowed to colonise a host blastocyst [8].

In the testis, germ cell development is maintained by GSCs known as A_{single} (A_s) spermatogonia that lie in contact with the basement membrane of the seminiferous tubule. The existence of niches in the testis was demonstrated directly when early germ cells were transplantated into the seminiferous tubules of host males whose GSCs had been depleted [10].

1.2.2.2 Adult Stem Cells

In the adult world, many developing tissues set aside life-long reservoirs of somatic stem cells, which retain some of the versatile characteristics of their early ES cells counterparts, including the capacity to seemingly end-lessly self-renew [9].

Adult stem cells are often relatively slow-cycling cells able to respond to specific environmental signals and either generate new stem cells or select a particular differentiation program. When a stem cell undergoes a commitment to differentiae, it often first enters

a transient state of rapid proliferation. Adult stem cells are often localized to specific niches, where they utilize many but not necessarily all, of the external and intrinsic cues used by their embryonic counterparts in selecting a specific fate [11].

1.2.3 Self-Renewal

Self-renewal potential is the most fundamental property of stem cells [4]. Self-renewal can be defined as making a complete phenocopy of stem cells through mitosis, which means at least one daughter cell generated by mitosis possesses the same capacity of self-renewal and differentiation [12].

What limits the number of stem cells under steady-state conditions? One possibility is that stem cells can only exist in a restricted microenvironment in each tissue, which provides factors that maintain them and excludes factors that induce differentiation [4]. If the amount of space in such microenvironments is limited, the number of stem cells would be limited by the number that can fit in that space. Stem cells generated in excess of the available space would differentiate. Not all stem cell systems, however, utilize such local control mechanisms [4].

1.2.4 Plasticity

Several reports suggest that there is far more plasticity than previously believed in the developmental potential of many different adult cell types. Examples of this surprising plasticity include the in vivo generation of murine skeletal muscle cells from bone marrow cells [13] and of bone marrow from skeletal muscle cells liver, kidney, heart, lung, etc. [14]. Neuronal and glial cells arising from bone marrow [15]. HSC into mature hepatocytes in the liver of rodents [16, 17], and this differentiation of bone marrow cells into mature cells of the liver also occurs in humans [17, 18].

If cells from diverse organs can migrate systemically, enter other organs and assume morphologies and functions typical of their new environment, then these changes in cell fate may not always be linear. In other words, there may be multiple sources of stem cells and routes whereby an organism can generate specific types of mature differentiated cells. Thus, the microenvironment, including contact with surrounding cells, the extracellular matrix, the local milieu as well as growth and differentiation factors, is likely to play a key role in determining a stem cell's function [12].

1.3 Bone Marrow Hematopoietic Stem Cells

Bone marrow contains hematopoietic stem cells producing all the blood cells, and mesenchymal stem cells. Moreover, transplantation of adult bone marrow cells can generated unexpected phenotypes in vivo, including, brain cells, muscle cells, liver

cells, and others. These data indicate that adult bone marrow might contain pluripotent stem cells, or stem cells that could become pluripotent under appropriate conditions.

Hematopoietic stem cells are lineage-uncommitted bone marrow cells that are characterized by the expression of cell surface markers, Sca-1 and c-kit, and the absence of lineage-specific markers for wide blood cells, red blood cells and platelets [19, 20]. They are rare populations representing 0.005–0.01 % of total bone marrow cell [19–21]. The pluripotency of hematopoietic stem cells is evidenced by their capacity to differentiate into multiple lineages within the blood and immune system, as well of non-hematopoietic tissues [22–24].

Because bone marrow-derived HSCs continually migrate into peripheral blood, and because this process can be enhanced by soluble factors, it would seem natural that such signals would be up-regulated in response to physiological or pathological tissue signal if hematopoietic cells had a role in the normal o abnormal repair process. Many investigators now believe the number of cells that undergo the transition from bone marrow stem cells to other tissues, may in fact represent a very small number and that the repopulation is rather low. Therefore, physiologically, in vivo, they may well be relatively rare events, although clearly demonstrable in vivo, play any significant role in the response of the human organs to tissue injury.

In the hematopoietic system, the properties of differentiation, multipotentiality, and self-renewal were first demonstrated more than 40 years ago through a series of seminal experiments demonstrating the ability of a subset of cells within the bone marrow (BM) to form macroscopic colonies on transplantation into the spleens of lethally irradiated recipient animals. Such colonies, termed colony-forming unit spleen (CFU-S), were found to contain differentiated progeny of multiple blood lineages [25], and a subset of these colonies could reform CFU-S when transplanted into secondary hosts [26]. Although originally believed to be derived from hematopoietic stem cells (HSCs), it is noteworthy that the CFU-S described by Till and McCulloch [25] were later found to be derived from more committed progenitor cells [27], thus providing an important lesson regarding the complexity of stem and progenitor cell biology.

HSCs were the first tissue-specific stem cells to be prospectively isolated [19] and are the only stem cells in routine clinical use to date. In the hematopoietic system, HSCs reside at the top of the hematopoietic hierarchy and give rise to functional effector cells of at least nine distinct types produced from HSCs in successive differentiation processes of increasingly committed progenitor cells. Because of the very short life span of most effector cells, mature blood cell production is an ongoing process with estimates suggesting the production of 1.5×10^6 blood cells every second in an adult human. This high turnover rate necessitates profound homeostatic control mechanisms, the primary level of which resides with the HSCs.

The immediate progeny of HSCs are multipotent progenitor cells that retain full lineage potential yet have a limited capacity for self-renewal. Multipotent progenitors in turn give rise to oligopotent progenitors, which possess more restricted developmental potential. Such oligo-potent progenitors in turn give rise to more lineage-restricted progenitors from which all of the mature blood cells eventually arise. Although questions remain regarding the absolute lineage potential of the

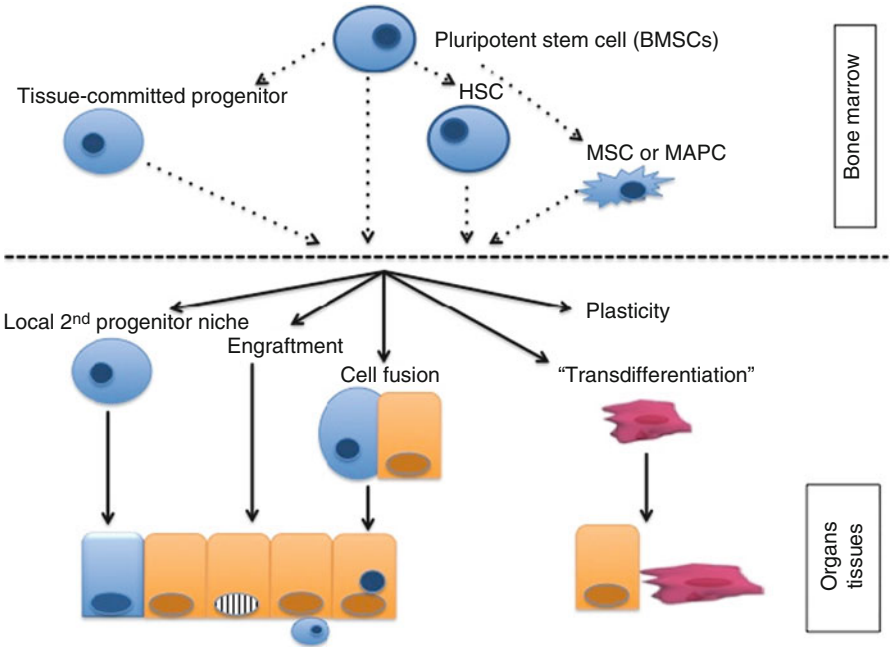

Fig. 1.1 Models of derivation from bone marrow cells. Several possible marrow cell types may serve as the source of epithelial cells. Four possible biological mechanisms may meditae tissue epithelial stem cells. These include: (1) trafficking of marrow cells to a local progenitor niche in the tissue, (2) fusion of bone marrow-derived cells with differentiated epithelial cells in the tissue, (3) direct "transdifferentiation" into tissue epithelial cells

different hematopoietic progenitor subsets and their relationship to one another, there is wide consensus that the sequential differentiation of HSCs through progenitors to fully differentiated blood cells is a primarily irreversible process under normal physiological steady-state conditions.

An increasing amount of data has suggested that many tissues are derived and maintained by small subsets of cells, commonly referred to as comitted stem cells, which exhibit many of the properties of normal tissuespecific stem cells including the capacity for unlimited self-renewal [28]. The extent to which stem cells are derived from tissue-specific stem cells themselves, or are the result of transformation events that imbue other cell types with stem cell-like properties, is currently under intense investigation and is likely to differ in different types of tissues.

1.4 Stem/Progenitors from Outside the Bone Marrow

A growing body of evidence has shown that tissues can be repaired by cells acquired via the circulation [29]. Similar cells were found throughout the body, in each case giving rise in vitro to the tissue from which they were isolated. It is thought that they lie dormant until activated by injury or disease (Fig. 1.1).

Recent progress in stem cell research indicates that certain mammalian cells, even from adults, maintain a high degree of plasticity for multilineage cell differentiation. This indicated that the extracellular factor(s) or cell-cell interaction might be sufficient for reprogramming adult cells into a more pluripotent status.

Bone marrow (BM) stem cells develop into hematopoietic and mesenchymal lineages but have also been known to participate in production of mature epithelial/stromal cells types. Evidence suggests that hematopoietic stem cells (HSCs) might have unexpected developmental plasticity.

All circulating blood cells, including myeloid cells, lymphocytes, and platelets are derived from hematopoietic stem cells (HSCs). In addition, hematopoietic stem cells are a source of tissue-residing cells. Bone marrow contains hematopoietic stem cells (HSCs) as well as mesenchymal stem cells and multipotent adult progenitor cells.

HSCs produce not only all of the blood lineages, but also liver and pulmonary epithelium, glia and neurons, skeletal and cardiac muscle, glomerular mesangial cells, overwhelm data lend credence to the possibility that a cell associated with the BMHSCs may act, under certain physiological conditions, as the progenitor of several types of tissue stem cells. Under specific physiological environment conditions, HSCs can differentiate into the all of mature of epithelial cells present in the tissue/organs. If resident bone marrow (BM) hematopoietic stem cells (HSCs) can form a variety of cell types, they may be more multipotent than the phrase *pluripotent hematopoietic stem cell* indicates.

Moreover, within bone marrow, only a rigorously *pure set* HSCs gave rise to adult-derived cells. This hypothesis seems to contradict the conventional assumptions of the germ layer origins of differentiated tissues, and raises the question of whether the cells of the HSCs phenotype are pluripotent HSCs that retain the ability to be the *universal stem cell source*. The hypothesis proposed was that (stem cells) migrating into tissues from the circulation that encountered a specific microenvironment responded to the signals found there (i.e. growth-factor-, cytokine-, or extracellular matrix-derived), by differentiation down certain lineage pathways.

Hematopoietic stem cells (HSCs) residing in adult bone marrow possess the unique ability to self-renew and differentiate into multiple lineages. In humans, HSCs are both necessary and sufficient for the lifelong regeneration of more than one million blood cells per second [30]. A number of recent studies have suggested that bone marrow cells or enriched HSCs, upon transplantation into adult recipients, might also transdifferentiate and contribute to the regeneration of a variety of non-hematopoietic lineages in multiple organs [30–33].

These findings have provoked extensive follow-up studies; some cast doubt on the biological significance and even the existence of such transdifferentiation [34], and other suggests alternative mechanisms for the observed developmental plasticity of transplanted bone marrow cells [35, 36].

1.4.1 Engraftment

It is well known that marrow derived mesenchymal and hematopoietic stem cells circulate for long periods after transplantation, allowing an equilibrium to be established between circulating and tissue-specific seeding compartments. It is

therefore conceivable that low-level recruitment of blood-bone precursors into the transplanted organ occurs in response to local events in the tissues microenvironment.

The level of engraftment of blood and marrow post transplant suggest that the expansion and differentiation of a single marrow SC to reconstitute the majority of the hematopoietic system of a lethally irradiated recipient is feasible.

The variable level of engraftment following single-cell transplantation is likely due to donor HSC concentration in the recovering host and the variations in successful homing to the marrow space, which is necessary for successful seeding of HSC.

Donor-derived epithelial cells were detected in lung, GI tract, and skin, and were distinguished from intraepithelial hematopoietic cells (i.e. lymphocytes, polymorphonuclear leukocytes, and macrophages) by their cytokeratin staining, morphology, and examination of parallel sections.

The epithelial engraftment was found at different frequencies in different organs. These differences may be due to (a) the degree of tissue damage induced by the transplant, (b) the residual tissue-specific stem cell capacity within each organ, and/or (c) the normal rate of cell turnover in each organ. These possibilities are supported by the variable levels reported for engraftment by marrow-derived cells.

Thus there are two patterns of epithelial engraftment of marrow-derived cells: large-scale repopulation in response to injury (as demonstrated in liver and lung) and low level engraftment as individual scattered cells in the absence of marked injury (e.g., liver, skin, and GI tract).

1.4.2 Transdifferentiation

Transdifferentiation is a poorly understood process invoked to explain how tissue-specific adult stem cells can generate cells of other tissues, acquiring broader development potential. Stem cells from one tissue (i.e. bone marrow) can circulate to another tissue and adopt the developmental fate of the second tissue.

Plasticity or trans-differentiation is a stem cell property by which a stem cell from a given tissue is able to generate differentiated cells from another tissues [22, 30] These differentiated cells usually have the same morphological characteristics and display the same surface markers as the remaining mature cells of tissue.

Transdifferentiation of an adult cell requires that it reprogram its nucleus based on extracellular signals. In different studies, transplanted stem cells show plasticity in vivo through their integration within a different tissue from its original tissue, acquiring at least some of its characteristics [13, 31–33, 37].

However, stem cell plasticity has been an issue of intense debate since this finding, and to date there are limited evidences that adult stem cells can generate mature, fully functional cells belonging to other tissues distinct from its original tissue [30]. In addition, in studies about transdifferentiation, it is essential to ensure the purity or homogeneity of the population of stem cells in the study, to exclude the possibility that different stem or progenitor cells may be contributing to the results. During the trans-differentiation, the original stem cell loses the tissue-specific markers and functions and acquires markers and functions of a transdifferentiated cell.

There are different studies [12, 22, 38] in which several mechanisms to explain the transdifferentiation/plasticity/lineage conversion have been proposed [34]. Thus,

lineage conversion may occur by activation of a differentiation program, otherwise dormant, to alter the lineage specificity of cells. Lineage conversion could also occur via de-differentiation of a tissue-specific cell to a more primitive, multipotent cell, and subsequent re-differentiation along a new lineage. Lineage conversion may also arise from a single and rare pluripotent adult progenitor cell, present in many tissues, which is able to generate tissues from multiple germ layers.

Four plasticity pathways have been documented in vivo and experimentally. These pathways may involve *undifferentiated* cells, situated within specialized tissues that can switch developmental programmes in response to stimulus. Another possibility is that differentiated cell types may *de-differentiate* to an earlier, progenitor phenotype. This process is probably more common in neoplasia. Alternatively, *differentiate* leaps can be induced by experimental manipulation or, in vivo, in response to stimulus. Thus, cells of differentiated phenotypes can have wide developmental ranges, and are not confined to the tissues from which they are derived. Finally, *fusion* between cells in some models can lead to reprogramming of nuclei.

1.4.3 Cell-Cell Fusion

Finally, it is possible to consider cellular fusion as a mechanism to explain stem cell plasticity. In cell fusion, generated stem cells inherit molecular markers and properties from both original stem cells. Thus, fused stem cells can contribute, as chimeras, to different adult tissues. This is especially relevant in cell types that normal fuse, as skeletal and cardiac muscle cells [35, 36].

Surprisingly, cell fusion was a frequent rather than rare event, in that up to 1 % of the hMSCs added to the coculture system were recovered as binucleated cells expressing an epithelial surface epitope. Some of the fused cells also underwent nuclear fusion. Two reports suggested that cell fusion can explain some of the observed plasticity of adult stem cells. The reports examined spontaneous fusion between embryonic stem cells and either unfractioned bone marrow cells, neural stem cells, or differentiated neural cells.

Importantly, several laboratories demonstrated that fusion of marrow-derived cells with recipient cells explain the colocalization of tracking and differentiation markers, rather than true stem cell plasticity [35, 36, 39–41]. Fusion of marrow-derived cells with organ cells has been documented in recipient liver, heart, and brain [39, 42]. On the other hand, the functional implications of fusion remain unclear and may conceivably be a potential mechanism for injury repair [42]. Fusion events, however, may not mediate all cell engraftment events [39, 43].

1.5 The Primary Niche

Though it was previously accepted that stem cells were tissue-specific, several studies have informed that this may not be the dogma and that the stem or precursor cell from one tissue can, under the appropriate conditions, differentiated into cells of another organ, giving rise the concept of stem cell plasticity. Previous finding of

blood-borne cells in the adult organs, and the characterization of a potential *universal stem cell source*, giving the rise to intact organ tissue, are challenging of the long-held views about the nature of the differentiate epithelium and its powerful to renew. It has been shown in experimental models that bone marrow derived stem cells can engraft in the adult organs and differentiate into mature cell epithelium.

On the other way, some researches appointed that fusion of marrow-derived cells with recipient cells explain the co-localization of tracking and differentiation markers, rather than true stem cell plasticity [36, 44, 45]. There is some debate as to whether the blood-borne cells that embed in the tissues actually differentiate to organs epithelium or fuse with cells in vivo. Furthermore, resident organ cells with features of marrow stem cell properties have been identified. However many question remain as to how such cells seed into the adult organs.

1.5.1 Tissue-Specific Adult Stem Cells

By definition, stem cells are self-renewing, slow-cycling, undifferentiated cells, with the potential to produce more than one type of differentiated cell. A division of a stem cell (SC) produces one stem cell (self-renewal) and one transit-amplifying cells (TA). TA cells are rapidly cycling progenitors, which mean that there is no phase of quiescence between successive cell cycles and the cycling time of TA cells do not exceed 3 days in vivo [46]. After a few divisions of TA cells, the progeny become permanently quiescent and fully differentiated.

Self-renewal results from asymmetric cell division with one daughter cell retaining all characteristics of the parental stem cell and the other destined to mature into an organ-specific cell type. Multipotency describes the ability of stem cells to differentiate along multiple cell lineages. It is well admitted that the bone marrow, and by extension adult organs harbour stem cells able to regenerate the cell types of that each organ.

1.5.2 The Niche Aging

Stem cells in different compartments share properties such as pluripotency, self-renewal, and diminished regenerative potential in old age. Several adult stem cells retain most of their intrinsic functional capacity throughout life. However, age-related changes in the environment surrounding the niche preclude their persistent activation. For example, the decline in stem cell number in old age is thought to be a result of an aging niche rather than an exhaustion of the stem cell pool.

In the bone marrow, it is the stem cell niche that regulates hematopoietic stem cells (HSC), maintains their quiescent state, and protects them from replicative senescence [47]. Aging of the niche leads to a significant reduction in the functional ability of HSC [48]. Likewise, aged skeletal muscle successfully regenerates when transplantated into a young host, while the regenerative capacity of young muscle is impaired

when transplantated into an aged host [49, 50]. Even in the liver, there is a well-documented decline in the proliferation of hepatic progenitor cells with age [51].

Our model presumes that, as the mammal aging become a decline in SC functionality takes place. This is due to the interplay between multiple extrinsic and cell-intrinsic factors that act to modulate key molecular pathways that control the regenerative potential of SC. Mechanistically, a wide range of pathways seem to contribute to SC aging and many of them connect one way or another to accumulating genotoxic stresses, as DNA damage, p53 activation, telomere shortening, c-kit decline expression, and prolonged aberrant expression of pluripotency genes (i.e. Notch, Oct4, SOX2).

Detailed analysis of stem cell niches in various systems reveals that stem cell activity is governed by supporting cells resident in the immediate vicinity of stem cells [52]. Support cells influence stem cell functions via direct physical interaction of membrane proteins, and also by the secretion of factors that bind to integral proteins expressed by stem cells and thus modulate their behavior [10, 53].

1.6 The Secondary Niche

The basic characteristic of an adult stem cell is a single cell (clonal) that self-renews and generates differentiated cells. The existence and localization of adult stem cells remains obscure: we expect that some cells in mature organs will turn out to be resident stem cells.

Is there a single progenitor (stem cell) for all organs cell types? If the origin or lineage of the stem cells in well understood for embryonic stem cells their origin in adult organs is less clear and in some cases controversial. The paucity of information on the developmental origin of adult organs stem cells leaves open the possibility that they to escape lineage restriction (in the early embryo) and subsequently colonize specialized niches, which function to both maintain their potency as well as restrict their lineage potential. Alternatively, the more widely believed, though still unsubstantiated, model for the origin of adult stem cells assumes that they are derived after somatic lineage specification, where upon multipotent stem cell-progenitors arise and colonize their respective organ niches (Fig. 1.2).

1.6.1 Bone Marrow Cells: Within Bone and Beyond

Despite progress in other areas of stemness, the complexities of this process remain poorly understood. Particularly enigmatic is the tissue-specificity of different niche types observed in human organs.

In 1989, Stephen Paget observed that circulating tumour cells would only “seed” where there was “congenial soil”. Meanwhile, the earliest changes occurring within distant tissues that prime the “soil” to receive incoming BMSCs have largely been neglected.

Dissemination of BMSCs is a prerequisite for niche replenish, but the two processes are not synonymous. Ceratin characteristics distinguish those cells able to

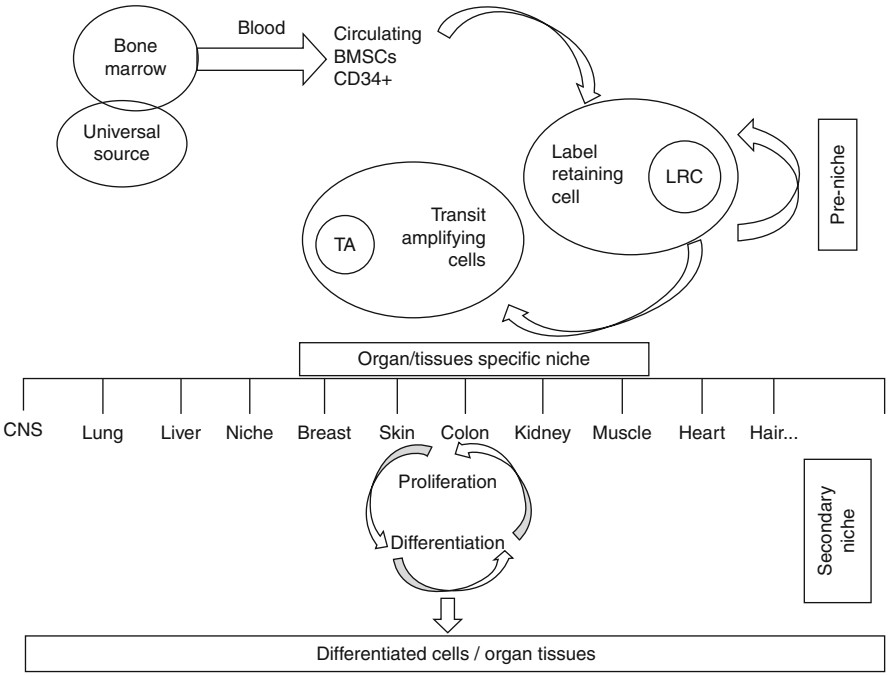

Fig. 1.2 Model for the origin of adult stem cells

colonize secondary tissues from other circulating BMSCs. For example a resistance to regulatory apoptosis is required for disseminating cells to evade the process of anoikis and amorphosis, cell death mechanisms induced by disruption of cell-cell or cell-matrix interactions and loss of cytoskeletal architecture.

1.6.2 Bone Marrow: Home for Hematopoiesis and Source for Migrating for Secondary Niche

The cell-specific genetic and phenotype make-up of a tumor is a major determinant of BM efficiency, but a receptive microenvironment is a prerequisite for establishing secondary BMSCs growth. The "poor prognosis" genetic profiles primarily include genes encoding cell surface receptors and secretory proteins, signifying the importance cell-stroma interactions.

Throughout ontogeny and in adult life, hematopoietic stem cells (HSC) migrate in a highly specific fashion, "homing" to regenerative sites (secondary niches) in the periphery or back to their niche within the bone marrow (BM). Migratory BMSCs utilize these physiological chemotactic and migration pathways in the process of repleshing empty secondary niches.

The dynamic interactions between BMSCs with the immediate microenvironments, of secondaries niches, are also alike. In the bone marrow and within the tumour

Table 1.1 Stem cells find their niche

Existence of LRC in secondary niche
Increase in the number of LRCs in secondary niche after injury
Descendants of LRCs function as TA cells during niche turnover
Immature mesenchymal phenotype of descendants of LRCs during niche turnover expresses vimentin
Differentiation of descendants of LRCs after proliferative phase expresses E cadherin

stroma, these niches constitute highly specific, physiologically defined, regulatory and supportive sites. Like a HSC within its niche in the marrow, and BMSCs must establish a relationship with host tissue stroma that is favorable to survival and growth at the secondary site. Unravelling the dialogue between HSC with secondary niche structures may help explain tissue-specific patterns of BMSCs observed between niche types.

1.6.3 Labeled Retained Cells (LRC)

In extensively studied tissues, LRCs reside in specialized and generally well-protected niches that are spatially proximal to their more differentiated progeny. These niches are believed to be a subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production [10, 54].

Of particular interest was that most of these were adjacent to capillary endothelial cells. In addition, LRCs were surrounded by vimentin-expressing cells after the cell proliferation phase had subsided. At this time, vimentin-expressing cells were no longer labeled with BrdU, presumably because multiple cell divisions caused BrdU staining to fade. There also observed that E-cadherin was expressed in differentiated/mature localized near the LRCs, suggesting the eventual differentiation of descendants to epithelial cells. Taken together, it is possible that LRC undergo asymmetrical cell division and that their descendants acquire immature mesenchymal phenotype as well as a high potential to proliferate and differentiate into epithelial cells and function as transit-amplifying cells during niche turnover regeneration.

LRCs phenotype remains unknown at present. Morphologically, LRCs were localized nearly mature cells and were co-localized with some markers, suggesting that LRC are in a differentiated state. However, these findings cannot totally exclude the possibility that LRC are undifferentiated (Table 1.1).

1.6.4 The Niche Stroma

The adult niche microenvironment has a poorly vascular network and a dense mesenchymal-derived stromal cell scaffold. The stromal matrix includes many essential growth factors, cytokines, chemokines and extracellular matrix components, that

regulate stem cells proliferation and differentiation, a process that can be maintained during physiological growth. Localization within the bone marrow dictates the fate and replication cycle of these cells. Differentiated cells generally reside in the vascularized niche closely associated with blood region, while primitive stem cells occur at the niche edge, closely associated with basal membrane (hypoxia place source). The stromal cells regulate and support SC, anchoring them to the niche in a state of quiescence. Endothelial stem cells (ESC) exhibit many similar phenotypic characteristics as the HSC in the bone marrow. The contribution of ESC to the niche vasculature is the uniquely specialized sinusoidal endothelial cells, which provide the second niche for BMSCs. Here, regulated proliferation, differentiation and maturation of migrating BMSCs occur.

1.7 Plasticity of BM Outside the BM

Some important considerations have emerged so far. First, the nervous tissue contains bona fide stem cells that support neuronal cell turnover throughout life. Second, despite their origin from one of the most quiescent tissues in the body, NSCs can undergo effective long-term culturing, proliferation and expansion while retaining stable functional characteristics. Third, when properly challenged, the overall developmental potential appears to be broader than that observed under physiological conditions in vivo [55].

One of the most intriguing cases is the virtual pluripotency of bone marrow-derived cells; however, multiple examples of Scs giving rise to cells normally found in other tissues have become available. In some cases, both the original SCs and the cells to which they give rise derive from the same embryonic germ layer (intragerm layer conversion). For instance, intramesoderm conversion has been documented by showing the genesis of skeletal or cardiac muscle cells from bone marrow cells. Similarly, muscle precursors can give rise to hematopoietic cells, although it has now been shown that the original muscle population undergoing conversion are Sca-1 and CD45-positive cells, which are hemopoietic in origin. Finally, muscle satellite cells retain an osteogenic and adipogenic differentiation potential that is normally retrieved in mesoderm-derived stromal cells. More striking examples of transgerm layer conversion – hereby also defined as transdifferentiation – in which SCs and their progeny belong to developmentally unrelated cell lineages have been reported [55].

1.7.1 Bone Marrow-Derived Cells Recruited to the Secondary Niche

Secondary niche cells secrete a multitude of chemokines and growth factors that induce changes in local stroma, and also direct the recruitment and proliferation of bone marrow derived cells to support new niche development.

BMSCs have been defined, by in vitro and in vivo studies, as pluripotential adult stem cells [56]. They posses the capacity to differentiate into different kinds of cells such as osteoblasts, chondrocytes, adipocytes, muscle cells, and neural cells [56, 57].

They are characterized by their high proliferative capacity ex vivo, whereas maintaining their ability to differentiate into multiple stromal cell lineages. The tissue-specific differentiation of BMSCs seems to be dependent on their state of differentiation and commitment, and the microenvironment in which they are located.

There have been shown that human bone is generated after xenogeneic transplantation of BMSCs with hydroxyapatite/tricalcium phosphate (HA/TCP) as a carrier vehicle [58]. The undifferentiated stem cell divides to generate transit-amplifying cells that further differentiate into postmitotic terminally differentiated cells that retain the SC phenotype in the "SC-TA-PM" scheme. The transit-amplifying cells in turn undergo proliferation and terminal differentiation to populate the tissue.

1.7.2 Marrow to Liver (Hepatocyte)

It has been shown in animal models that hepatocytes, biliary cells, or oval cells during liver regeneration can derive from bone marrow cells [30]. Two reports have indicated that the bone marrow of adult rodents contains progenitor cells with the potential to give rise to cells expressing the hepatocytes markers cell-cell adhesion molecules [16, 59]. The existence of a bipotent hepatic progenitor or stem cell, capable of regenerating both hepatocytes and cholangiocytes in response to injury, has long been investigated and there now appears to be consensus that such a cell population does exist [60–64]. Such a process occurs in humans and therefore shows that in humans, hepatocytes and cholangiocytes can be derived from extrahepatic circulating stem cells, probably of bone marrow origin, and such transdifferentiation can replenish large numbers of hepatic parenchymal cells.

Circulating, marrow-derived cells might enter the liver cell plates directly from the sinusoidal circulation. Incorporation of mature hepatocytes into the liver cell plates after vascular injection has been already reported [65].

The experiment of Theise et al. [59] show that bone marrow cells transplanted from male donors to syngeneic recipients are able to localize in the two largest lobe of the liver, differentiating into mature hepatocytes.

The presence of a facultative liver stem cell compartment has been documented in both animals [62, 64] and humans [66, 67].

Petersen et al. [16] reported hepatocyte differentiation of transplanted bone marrow cells after acute, severe liver injury. In our studies showing bone marrow-derived hepatocytes, there is minimal, if any liver injury, indicating that stem cells of bone marrow origin may take part in normal tissue renewal in the liver.

1.7.3 Marrow to Muscle Cardiomyocites

The data of Deb et al. suggest that adult human bone marrow acts as a source of extracardiac progenitor cells contributing to cardio-myocite formation and muscle [13, 68].

Ferrari et al. [13] demonstrating skeletal muscle differentiation of injected bone marrow cells in response to myocite injury, and suggesting that bone marrow-derived cells might function as progenitor cells for unexpected tissues. The potential origin and phenotype of marrow myocite precursors in our subjects includes lineage-restricted mesenchymal [69], hematopoietic [33], and multipotent adult progenitors [69] and cells of angioblastic lineage [70].

1.7.4 Marrow to Lung

The recent finding blood-borne cells in the lung and the characterization of a potential universal stem cells, giving rise to intact lung tissue, are challenging some of the long-held views about the nature of the pulmonary epithelium and its capacity to renew. It has been shown in experimental models that bone marrow-derived cells can engraft in the lung and differentiate into mature epithelial phenotypes [44, 71] and that this process increases in response to injury [72].

There is some debate as to whether the blood borne cells that embed in the lung actually differentiate to lung epithelium of fuse with cells in situ, as he has been shown to occur in vitro [45]. The presence in the lung epithelium of cells recruited from the circulation could provide new opportunities for a range of pulmonary diseases by providing means to repair the lung and novel route for gene therapy. For the human lung, chimerism has been demonstrated in pulmonary epithelium, including that of the alveoli, following transplantation of haematopoietic stem cells or lung [73], although neither study found evidence for engraftment of bone marrow cells specifically.

1.7.5 Marrow to Brain (Astrocytes)

Two different systems show that BM-derived stem cells can serve as progenitors of nonhematopoietic cells in the murine central nervous system (CNS). In one study, lethally irradiated adult mice that received whole marrow intravenously developed donorderived brain cells bearing the neuronal antigens NeuN and class 3 b-tubulin [31]. In a separate study, after marrow cells were injected into nonirradiated newborn mice, they migrated to the brain where they expressed NeuN [32].

1.7.6 Marrow to Kidney (Glomerular Mesangial) Cells

The hypothesis that mesangial cells are derived from hematopoietic stem cells, were demonstrated from a single experiment that unequivocally establish the hematopoietic origin of glomerular mesangial cells [74]. Previously there are reported that bone marrow (BM) cells can reconstitute mesangial cells in the kidney of lethally

irradiated mice [75]. These processes, demonstrating that a single hematopoietic stem cell is capable of differentiating into glomerular mesangial cells, dowes not involve cell fusion.

1.7.7 Marrow to Skin

BMSCs may engraft as epithelial and endothelial cell types within the healing wound [76]. Ongoing studies in our laboratory have demonstrated that BMSCs engraft as proliferating ($Ki67^+$) keratinocytes at the wound edges, then migrate to the wound area and become part of the scar tissue. These findings suggest that BMSCs contribute to wound healing.

1.7.8 Marrow to Gastrointestinal Tract

Injecting a single marrow-derived stem cell with long-term repopulating ability in mice leads to low numbers of donor-derived esophageal and bowel epithelial cells [44]. Unlike BMSCs, MAPCs administered intravenously can engraft as GI crypt cells, the functional stem cells of the gastrointestinal (GI) epithelium [69]. BMSCs also engraft as epithelial cells in the human GI tract after allogeneic BM transplantation [70].

Conclusions

Tons of articles exist about the existence of pluripotential stem cells that can give rise to any cell of the body and that are present in the different tissues. Moreover, it is believed that these cells would be responsible for replenish adult stem cell niches.

Whether stem cells exist or not, what it stands for clear is that tumor resistance to current anti-cancer therapies may rely on stromal cells with the ability to avoid and escape for current local and systemic armamentarium.

There is still a long way in the process of understanding the tumor behaviour and the isolation of tumor stem cells that are key drivers for malignant spread and development.

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Chapter 2
What Is the Meaning of Notch Pathway and How Can We Selectively Do the Targeting?

Ana Custodio and Jorge Barriuso

Abstract Notch receptors participate in a highly conserved signaling pathway that regulates normal developmental and tissue homeostasis in a context-dependent manner. Deregulated Notch signaling is involved in numerous human diseases and recently, a substantial body of evidence has been generated in support of this pathway playing critical roles in several types of cancer. The finding that activating Notch-1 mutations are frequently found in patients suffering from T-cell acute lymphoblastic leukemia is one of the best examples, but an abnormal expression of different human Notch receptors also contributes to B-cell tumors as well as a number of solid cancers such as breast, colon, pancreas, brain, lung, skin and other tissues. Several **γ**-secretase inhibitors are currently being explored for their potential therapeutic applications in Notch-associated tumors. Alternative approaches involve the development of antibodies to inhibit Notch receptors, their activating ligands, or other components of the Notch pathway. In this book chapter, we review the rationale for Notch inhibition in cancer, the current state of the art, as well as potential strategies that try to target the oncogenic properties of Notch signaling.

Keywords Delta-like • Gamma-secretase • Inhibitors • Jagged ligand • Notch • Stem cells

2.1 Introduction

Notch pathway was first recognized as an important developmental pathway in Drosophila in the first half of the twentieth century [1]. Several decades later, it was shown this pathway powerfully influences stem cell maintenance, differentiation and cell fate decisions [2–5]. In the last years, a number of preclinical but also

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clinical studies have shown that Notch signaling plays important roles in numerous human diseases, including a broad spectrum of malignancies [6–10]. Therefore, the Notch pathway has a tremendous potential as a new target in cancer therapy and there is growing evidence that synergy can result from combining Notch inhibition with already existing treatment modalities such as chemotherapy (CT), radiation and other pathway inhibitors [11–15]. This book chapter will address the current knowledge of Notch signaling in various types of hematological and solid tumors as well as potential therapies that try to target its oncogenic properties.

2.2 Notch Signaling Pathway

As introduction, it is important to consider the complex studies of this highly conserved signaling pathway. The Notch pathway is a short-range communication system in which contact between a cell expressing a membrane-associated ligand and a cell expressing a transmembrane receptor sends the receptor-expressing cell (and possibly both cells) a cell fate regulatory signal. This signal takes the form of a cascade of transcriptional regulatory events, that involves the expression of hundred if not thousands of genes, and has profound context-dependent phenotypic consequences [16].

Mature Notch receptors are large single pass transmembrane noncovalent heterodimers consisting of an extracellular subunit and a transmembrane subunit. Whereas the fly genome contains only one Notch receptor, and worms have two that act redundantly [17], mammals have four paralogs (Notch-1, -2, -3, and -4) that display both redundant [18] and unique functions [19]. The extracellular domain of all Notch proteins contains 29–36 tandem epidermal growth factor (EGF) -like repeats, some of which mediate interactions with ligands. The activating interaction with ligand presented by neighboring cells (*trans* interactions) are mediated by repeats 11–12, whereas inhibitory interaction with ligand co-expresses in the same cell (*cis* interactions) are mediated by repeats 24–29 [20]. Many EGF repeats bind calcium, which plays an important role in determining the structure and affinity of Notch to its ligands [21, 22]. The EGF repeats are followed by a unique negative regulatory region (NRR) composed of three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (HD). The NRR plays a critical role in preventing receptor activation in the absence of ligand. The single transmembrane domain (TMD) is terminated by a “stop translocation” signal comprised of 3–4 Arg/Lys residues. Intracellulary, the RBPjκ association module (RAM) domain forms a high affinity binding module of 12–20 aminoacids centered on a conserved WxP motif [23]. A long unstructured linker containing one nuclear localizing sequence (NLS) links RAM to seven ankyrin repeats (ANK domain). Following the ANK domain are an additional bipartite NLS and a loosely defined and evolutionarily divergent transactivation domain (TAD). The very C-terminus contains conserved proline/glutamic acid/serine/threonine-rich motifs (PEST), which regulate the stability of the Notch intracellular domain (NICD).

Most canonical Notch ligands are similar but smaller single-pass transmembrane proteins, that are characterized by three related structural motifs: a specialized delta/serrate/lag-2 (DSL) domain at the N-terminus, a specialized tandem of EGF repeats called the DOS domain (Delta and OSM-11-like proteins), and EGF-like repeats [24, 25]. Both the DSL and DOS domains are involved in receptor binding, with the DSL domain involved in both *trans* and *cis* interactions with Notch. Notch ligands can be classified based on the presence/absence of a cysteine-rich domain (Jagged/Serrated vs. Delta, respectively) and a DOS domain [25]. There are three Delta-like proteins (DLL-1,-3, and -4) and two Jagged proteins (JAG-1 and -2), which are differentially expressed in different cells. Other ligands have been proposed, such as the NOV (nephroblastoma-overexpressed), the neural adhesion molecule F3/contactin [26], the related NB-3 protein [27] and the EGF repeat protein DNER [28], but they have not been as well-established as Delta-like and Jagged.

Signal transduction by Notch receptors relies on a series of proteolytic cleavages. During trafficking to the cell surface, Notch is cleaved by a furin-like protease at a site termed S1 located within an unstructured loop protruding from the HD domain. As a result, mature Notch receptors are heterodimers made up of non-covalently associated extracellular and transmembrane subunits that are held together by the intrinsic stability of the HD domain [29]. Signaling is initiated when Notch receptor of one cell binds to a Notch ligand expressed on a neighbouring cell. This set in motion events that lead to the cleavage of Notch by two additional proteases. The first cleavage is carried out extracellularly by an alpha-secretase (a disentegrin and metalloprotease ADAM-10 and ADAM-17) at site 2 (S2), which is located 12–14 amino acids external to the TMD [30, 31]. In the resting state, the S2 site is deeply buried within the NRR, indicating that a substantial conformational change must precede S2 cleavage. S2 cleavage requires the endocytosis of ligand into the ligand-expressing cell, leading to speculation that mechanical forces transmitted to the NRR via endocytosis are responsible for the intramolecular movements that precede S2 cleavage [32]. The shedding of the Notch ectodomain creates a membrane-tethered intermediate, the Notch extracellular truncation (NEXT), that is cleaved within its transmembrane domain by γ-secretase, a multi-subunit protease consisting of presenilin 1 or 2, PEN-2, APH-1, and nicastrin. The ultimate cleavage at the site S3 frees the intracellular domain of Notch (NICD) from the membrane, allowing it to translocate to the nucleus [33]. Whether γ-secretase cleavage occurs at the cell surface or following endocytosis within endocytic vesicles remains controversial.

NICD contains several protein-protein interaction domains, including seven iterated ankyrin repeats (ANKs) that are needed for all known Notch functions and a so-called RAM domain. Nuclear NICD associates with the DNA-binding protein CSL (for CBF1, Suppressor of Hairless, Lag-2; also known as RBP-Jκ) through high-affinity RAM contacts and lower-affinity ANK contacts. In humans, CBF1 is the only CSL factor identified to date. Binding of ANK to CSL creates an extended, composite surface groove that recruits scaffold proteins of the Mastermind-like (MAML)/Lag-3 family, the third component of the core Notch transcription complex (NTC). The NTC in turns interacts with chromatin modifying factors such as the histone acetyl-tranferases p300 and pCAF, as well as components of the mediator complex, to transactivate

target genes. In the absence of NICD, CSL can associate with multiple proteins that suppress transcription, including multiple complexes with histone deacetylase activity and other factors with histone demethylase activity [34, 35]. For this reason, CSL has been likened to a molecular switch capable of actively suppressing or stimulating transcription, depending of the Notch activation status of a cell. Normal Notch transcription complexes are believed to have a half-life of the order of minutes, in part due to the presence of a PEST degron domain in the C-terminus of NICD that marks activated Notch for ubiquitination and proteasomal degradation, thus terminating the signal [16]. Notch target genes are numerous and include HLH-family negative transcriptional regulators of the Hes and Hey family, but also cell cycle progression genes (c-Myc, cyclin D1), antiapoptotic genes (Bcl-2), and many others yet to be discovered [36, 37].

Other aspects of the Notch pathway are also noteworthy. The Notch ligands Delta-like and Jagged are processed in a similar fashion to Notch following binding; they are cleaved also by alpha- and gamma-secretase, liberating an intracellular domain that is believed to be localized to the nucleus [38, 39]. The functions of their intracellular domains remain unknown. The likelihood of independent functions for Delta-like and Jagged proteins should be considered in the development of Notch inhibitors, as most strategies for Notch inhibition are also likely to inhibit Delta-like and Jagged. In addition, putative non-canonical pathways have been suggested but remain incompletely characterized. Among them, physical interaction of the intracellular domain of Notch-1 with the IKK signalosome, with nuclear IKKα and with p50. And the intracellular domain of Notch-3 with cytoplasmic IKKα may mediate therapeutically relevant cross-talk with nuclear factor κB (NF-κB) [40–42]. Physical interaction of the intracellular domain of Notch-1 with p85 PI3-kinase α may [43] mediate non-nuclear cross-talk with AKT, leading to survival signaling [44].

A closer look at this apparently simple signaling pathway reveals a intricate series of mechanisms that finely regulate the timing, intensity, and biological consequences of Notch signaling, and are likely to have significant therapeutic implications. Because the pathway relies on protein-protein interactions and the NICD is short-lived, a single activated Notch receptor is likely to transactivate only one target gene for a short period of time (on the order of minutes), a “design” that enables very precise temporal and quantitative control.

2.3 Notch Pathway Functions in Normal and Cancerous Cells: Rationale for Notch Inhibition in Cancer

The evolutionary conserved Notch signaling pathway functions as a mediator of short-range cell-cell communication. Numerous functions have been attributed to Notch, with some of these helping to explain its cancer-promoting effects in many tissues. However, Notch function can substantially differ and be dependent on cell type and tissue, and often the role of Notch signaling in a given tissue is unpredictable. Notch is among the most central pathways in self-maintenance of stem cells, along with

Hedgehog, Wnt, and perhaps transforming growth factor-β (TGF-β). Interestingly, Notch signals select among preexisting cellular potentials; in a context dependent manner they will either promote or suppress proliferation, cell death, acquisition of specific cell fates and activation of differentiation programs throughout development and during maintenance of self-renewing adult tissues [2–5, 45–56]. Notch has been found to be critical in development of the brain, heart, vasculature, fat, hematopoietic system, gut and immune system. For example, it drives toward a glial cell fate in the central nervous system (CNS) [45] and regulates the T helper 1 versus T helper 2 decision in the immune system [4]. Notch has two main physiological roles in the intestine. One is to maintain the proliferating undifferentiated SC/progenitors acting as a gatekeeper of crypt cells and the other is to monitor binary cell fate decisions of the transient amplifying compartment, resulting in increased proliferation of absorptive enterocytes and a severe reduction of all secretory cells-goblet, enteroendocrine, and Paneth cells [5, 46]. Notch signaling, and in particular, RBP-J, is also an important regulator of pancreatic progenitor cells that have to choose between the endocrine and the acinar cell fate [47, 48]. How Notch regulates this cell fate decision is not fully understood. One possible mechanism suggested that Hes-1(hairy/enhancer of split 1) represses the expression of neurogenin3, which functions as a pro-endocrine factor, and the cell-cycle regulator p57 [49], thereby preventing progenitor cells from exiting the cell cycle and from differentiating into the endocrine lineage. In addition, some reports confirm that although Notch is not very active in homeostatic conditions, it seems to play important roles during tissue regeneration [50]. Interestingly, a physiological role for Notch signaling in the melanocyte lineage has also been demonstrated. Notch acts through Hes-1 and plays an indispensable role in the maintenance of melanocytic stem cells and melanoblasts in the epidermis. When Notch-1 and/or Notch-2 are ablated in the melanocyte lineage, a diluted initial hair pigmentation at birth and premature hair graying in subsequent hair cycles is observed [51, 52].

As referred above, Notch signaling can also induce terminal differentiation, which is accompanied with growth suppression. The skin is one of the best-studied examples of Notch exerting growth suppressive functions. The epidermis is composed of multiple layers of keratinocytes that are separated from the dermis by a basement membrane. Skin stem cells (SC) and transient amplifying cells are found within the basal cell layer of the epidermis and they are the reservoir for epidermal SC. Keratinocytes that undergo cell-cycle arrest detach from the basement membrane and move upward to form a supra-basal spinous layer and post-mitotic keratinocytes continue to migrate toward the outer surface of the skin to form the granular layer. Notch-1, Notch-2, and Notch-3 mRNA are highly expressed in the basal cell layer and to a lesser extent in the suprabasal layer of the human epidermis [53]. DLL-1 expression was shown to be highest in regions where potential SC reside which led to the suggestion that DLL-1 mediated Notch signaling induces SC to differentiate into transient amplifying cells. Cell culture experiments combined with genetic mouse studies suggest that Notch signaling induces terminal differentiation processed in keratinocytes [53, 54]. The p63 gene is important for the self-renewing properties and stratification of keratinocytes in the skin. P63 is expressed in the proliferating compartment of the skin and is downregulated as soon as keratinocytes start to

differentiate. Notch-1 and p63 negatively regulate each other and thereby regulate the balance between self-renewing and differentiation [55]. In the context of cancer, p63 is often upregulated in epithelial tumors, including squamous cell carcinomas, in which Notch receptor expression is often downregulated [56]. Additional Notch-mediated mechanisms that help keratinocytes to differentiate are the induction of keratin1/10 and involucrin, as well as downregulation of integrin expression [54].

Because Notch plays a critical role in many fundamental processes and in a wide range of tissues, it is not surprising that aberrant gain or loss of Notch signaling components have been directly linked to multiple human disorders, from developmental syndromes, such as the Tetralogy of Fallot, Alagille syndrome, familial aortic valve disease, spondylocostal dysostosis or syndactyly [57], to adult onset diseases such as CADASIL (cerebral autosomal dominant artheriopathy with subcortical infarcts and leukoencephalopathy), Alzheimer's disease [58], and cancer [6–10].

As we referred above, Notch is one of the most powerful of the stem cell-promoting pathways, making it relevant for cancer given the undifferentiated/de-differentiated state of most tumor cells. The growing evidence for the "cancer stem cell" (CSC) hypothesis may make Notch a particularly exciting target in Oncology. This hypothesis states that cancers harbor a usually small subpopulation of pluripotent "CSC" or "cancer-initiating cells" that retains SC character and gives rise to the bulk population of cancer cells through a process of aberrant differentiation that recapitulates that of normal tissues. Such cells have now been isolated and cultured from leukemias, breast cancers, glioblastomas, and many other cancers. They are characterized by properties of normal SC, such as indefinite self-renewal through asymmetric cell division [59, 60] but also the ability to differentiate into cells resembling normal cell types in a given tissue, very slow proliferation rates and resistance to standard treatments such as CT and radiation, owing, in part, to overexpression of ABC export pumps and cell cycle checkpoints proteins [59, 61, 62]. Whether CSC are derived from the malignant transformation of normal tissue SC or from the "dedifferentiation" of normal non-SC is a matter of considerable debate. What seems likely is that these cells are uniquely capable of resisting anticancer agents, surviving for a long time in a nearly quiescent status and eventually cause disease recurrences and/or metastasis after apparently complete remissions. Thus, a complete eradication of them will be necessary to attain a cure. This will require targeting of pathways that participate in the survival, replication and differentiation decisions in pluripotent cells, such as the Notch pathway [59, 63–65].

Some of the impact of Notch inhibition in cancer cells results from its extensive cross-talk with several essential proteins and pathways in tumorigenesis [66–74]. Notch regulates expression of important receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor-1 (VEGFR-1) [66, 67] and also interacts with fibroblast growth factor receptor (FGFR) signaling [68]. It has been also demonstrated that Notch activity sustains the phosphatidyl inositol 3-kinase (PI3K)/Akt/mTOR pathway and is a mediator of the oncogenic function of the RAS/MAPK pathways [69–71]. In addition, Notch and the nuclear factor-κB (NF-κB) pathway are intimately related, with multiple points of interaction described [72], and the Myc oncogene is a direct target of Notch, mediating much of the oncogenic effects of Notch in T-cell malignancies [73]. In some instances, other oncogenic pathways have been shown to cross-talk with

Notch or its downstream activity, as is the case for the hypoxia/hypoxia inductible factor-1α (HIF-1α) pathway [74]. It is important to note that some of the described interactions are context-dependent and do not occur in all cellular backgrounds.

The direct effects of Notch inhibition on cancer cells may vary. Since Notch activity promotes cell survival and has anti-apoptotic functions through its interaction with important anti-apoptotic pathways such as Akt, it is not surprising that Notch inhibition has most frequently been shown to trigger apoptosis in cancer cells [70, 75]. Notch inhibition has also been shown to slow cancer cell proliferation and there is some evidence indicating that there may be important roles for Notch in the cell cycle in some settings [76]. Senescence has also been linked to the Notch pathway as its mediator, Hes-1, has been shown to play a critical role in blocking senescence [77]. Finally, the interaction between cancer cells and the surrounding stroma is receiving increasing attention as a key factor in tumor progression. "Tumor stroma" includes endothelial cells, necessary for tumor angiogenesis, fibroblasts that can produce growth factors and cytokines, as well as many subtypes of immunocytes, from T cells to dendritic cells or NK cells. There is significant evidence that bidirectional intercellular communication involving Notch signals takes place between tumor cells and stromal cells in some malignancies, suggesting that targeting the Notch-ligand interaction in endothelial cells can have therapeutic implications. For example, Notch blockade can impact the angiogenesis process [78–83]. The precise mechanisms by which Notch regulates the vasculature seem to be diverse. Notch/DLL-4 signaling directly regulates angiogenic endothelial cells and Notch also seems to regulate aspects of vascular development such as arterial versus venous fate [78]. Moreover, Notch also regulates the expression of the VEGFR-1, a key receptor for vascular formation. A number of reports have shown direct antiangiogenic effects from Notch inhibition [67]. A major role for Notch in blood vessels is supported by the vascular nature of the defects in the human disease called CADASIL syndrome caused by Notch-3 mutations [51]. Recently, multiple groups have found that signaling via the Notch ligand DDL-4 regulates endothelial sprouting and its inhibition lead to disordered and unproductive endothelial growth and decreased tumor size, even in tumor with vascular endothelial growth factor (VEGF) inhibition resistance [79, 80]. In addition, in mice the knockouts of Notch-1, DDL-1 or JAG-1 are embryonic lethal due principally to vascular defects [81] and small-molecule Notch-inhibiting drugs have been shown to have potent antiangiogenic effects in cancer animal models [82, 83].

2.4 Notch Signaling in Hematologic and Solid Tumors

2.4.1 Notch Signaling in T-Cell Acute Lymphoblastic Leukemia (T-ALL)/Lymphomas

Notch-1 was discovered in 1991 through analysis of T-cell lymphoblastic leukemias/lymphomas (T-LL) with balanced (7;9) translocations [84]. The translocation breakpoint were shown to fall within Notch-1 on chromosome 9 and the T-cell

receptor β (TCRβ) locus on chromosome 7 and to result in fusion of the 3'end of Notch-1 to TCRβ enhancer/promoter elements, which drive the expression of aberrant Notch-1 transcripts encoding truncated, constitutively nuclear Notch-1 polypeptides. Expression of similar forms of Notch-1 in murine hematopoietic stem cells (HSC) induces the appearance of T-LL [85, 86], whereas no tumors are observed when the same polypeptides are expressed in HSCs with genetic defects that abrogate T-cell development [87]. These studies revealed that Notch-1 had a special oncotropism for T-cell progenitors and it is both necessary and sufficient for T-cell development from HSCs [88].

The degree of Notch-1 involvement in human T-LL became apparent in 2004, when Notch-1 gain-of-function mutations were found in roughly 60 % of primary human T-LL [6]. The most common Notch-1 mutations in human T-LL consist of point substitutions or small in-frame insertions or deletions [29]. In 40–45 % of T-ALL, mutations perturb the NRR domain, leading to ligand-independent signaling or increased sensitivity to ligand. Other Notch-1 mutations cause displacement of the extracellular ADAM cleavage site away from the NRR domain, or shifting of the NNR domain away from the transmembrane domain, in both instances leading to de-regulated ADAM cleavage at the S2 site which results in ligand-independent proteolysis [29, 89]. A second type of Notch-1 mutations results in truncation of the C-terminal PEST domain, leading to increased stability of NICD and hence prolonged transcriptional activation of Notch-1 target genes [6]. Remarkably, individual tumors may harbor as many as three Notch-1 mutations, typically aligned in *cis* in a single allele and mutations abrogating NRR function are often found together with PEST domain mutations, indicating that T-cell transformation is driven by selection for increasingly high levels of Notch activity. Consistent with this observation, Notch-1 mutations have been detected as secondary events in T-cell subclonal populations in T-ALL patients [90]. Finally, given the strong selection for Notch-1 gain of function in T-LL and the ability of leukemic blasts to proliferate in many tissues where access to ligand is likely limited, it is likely that other mechanisms of ligand-independent Notch-1 activation remain to be discovered in human T-LL. This is particularly true of tumors that only have PEST deletions, as such mutations may have little or no effect on Notch-1 signaling in the absence of some mechanisms (e.g. an NRR mutation) that promotes Notch-1 proteolysis [6, 91].

In the context of transformed T-cell progenitors, Notch signaling induces and reinforces a programme of gene expression that supports cell growth. The most important direct target genes include c-Myc [6, 92, 93] and Hes-1 [6, 92, 94]. A key pathways activated by Notch-1 include the PI3K/Akt/mTOR, since Notch-1 signaling via Hes-1 down-regulates PTEN, an important negative regulator or PI3K/Akt signaling [71, 95, 96]. Notch-1 also up-regulates the expression of the interleukin-7 (IL7) receptor, which activates PI3K/Akt signaling in an IL7-dependent fashion. In culture systems, the growth of primary human T-LL cells is IL7-dependent, suggesting that the IL7/IL7-receptor signaling axis is an important mediator of growth [97, 98]. Notch-related T-LL is also associated with constitutive activation of NF-κB [40] and abnormalities of E2A [99], but the mechanistic bases for these relationships are not fully elucidated. In addition to Notch-1, developing thymocytes also

express Notch-3, which appears to be a downstream target for Notch-1 [6, 92]. Transgenic mice expressing N3ICD develop T-LL, but the relative importance of Notch-3 compared with Notch-1 has been uncertain [87].

Despite the confirmed role of Notch-1 mutations in all genetic and clinical subtypes of human T-ALL, associations between mutation status and outcome have been inconsistent. While a few series have suggested that Notch-1 mutations are associated with worse outcomes [100], most have shown no association or a trend towards more favourable responses [6, 101, 102]. Although Notch signaling has been linked to resistance to glucocorticoids in studies of T-LL cell lines [103], no such association has been found in primary tumors; in fact, in some series the trend is towards better responses to glucocorticoids among tumors with Notch-1 mutations [104].

2.4.2 Notch Signaling in Solid Tumors

The Notch pathway activation is a common step in the initiation and/or progression of many different human cancers, including breast cancer [105–118], colon cancer [119–123], pancreatic cancer [124–129], medulloblastoma [130–135], melanoma and other cutaneous tumors [136–148], Ewing's sarcoma [149], Kaposi's sarcoma [150], osteosarcoma [151], lung cancer [152], hepatocellular carcinoma [153, 154] or ovarian cancer [155, 156].

2.4.2.1 Breast Cancer

The first evidence describing a link between aberrant Notch signaling in solid tumors came from the observation in animal studies that the integration of the mouse mammary tumor virus (MMTV) into the Notch-4 gene leads to the formation of mammary tumors [105]. Over the past years several mouse studies have establish the role of Notch signaling during the normal mammary gland development [106, 107] and correlative evidence has also been accumulated implicating this pathway in human breast cancer. Activated forms of Notch-1 and Notch-4 have been identified in several human breast cancer cell lines [8] and Notch-3 has been shown to play an important role in the proliferation of ErbB2-negative breast tumor cell lines.

The first clue that Notch might be aberrantly expressed in primary human breast cancer came from a study demonstrating increased expression of Notch-1 in four breast cancer tumors that overexpressed H-ras identifying Notch-1 as a downstream target of oncogenic H-Ras [109]. Interestingly, tumor samples expressing high levels of both Notch-1 and its ligand JAG-1 are associated with particularly low overall patient survival rates, suggesting a synergistic effect of these expression level changes on tumor progression [110]. Increased accumulation of N1ICD and Hes-1 expression in ductal carcinoma in situ (DCIS) compared with normal breast tissue also predicts a reduced time to recurrence 5 years after surgery [111]. This finding confirms that both the accumulation of NICD as a useful prognostic marker for

recurrence as well as changes in the Notch signaling pathway may be associated with the progression from DCIS to invasive disease. Consistent with these observations, activated Notch signaling [8, 112] and consequent upregulation of genes that promote tumor growth [128–130] have been observed in breast cancer cell lines and primary breast cancers. In particular, Notch-4 expression, as detected by immunohistochemistry, correlated with Ki67 in infiltrating breast carcinoma of ductal or lobular histologies [112]. Activation of Notch signaling in estrogen receptor (ER)-negative breast cancer results in direct transcriptional up-regulation o the apoptosis inhibitor, and cell-cycle regulator survivin [113] and levels of Slug, a transcriptional repressor and Notch target, are elevated and correlate with increased expression of JAG-1 in human breast cancers [114].

On the other hand, Notch signaling plays a role in breast CSC and two recent studies have demonstrated that the Notch pathway is important for promoting the commitment of mammary stem cells to the luminal lineage at the expense of the myoepithelial lineage in both man and mice [116, 117]. The Notch pathway in vivo appears to be preferentially active in mammary luminal cells, with prominent expression of the active form of Notch-1 and its target genes (Hey1 and Hey2) in luminal progenitor cells. Expression of N1ICD in mammary stem cell promoted luminal cell-fate specification at the expense of the myoepithelial lineage. The constitutive overexpression of N1ICD led to specific expansion of luminal progenitors and their self-renewal, finally leading to hyperplasia and tumorigenesis [116]. In contrast to the mouse, where Notch-1 is the key determinant of luminal fate selection [117], Raouf et al. have showed that Notch-3 is critical for the restriction of bipotent progenitor cells to the luminal pathway in human breast tissue and that other Notch receptors could not substitute for this activity. Nevertheless, they showed that Notch-4 gene expression is highest in undifferentiated human clonogenic mammary progenitor cells, becoming markedly downregulated when these cells committed to the luminal lineage [116]. This finding is interesting in light of a study by Harrison et al., who have identified the activated form of the Notch-4 receptor in basal CD44+ breast CSC cell lines and in primary human samples, whereas N1ICD was observed at higher levels in the luminal cells of normal breast epithelium [118]. The differential distribution of Notch-1 and Notch-4 in basal CSC and more differentiated cells would suggest different roles for each receptor. Therefore, targeting the Notch-4 receptor specifically might be a feasible therapeutic approach. In summary, the luminal progenitor cell can be implicated as a potential cell of origin for tumors in which the Notch pathway has been activated inappropriately, leading to hyperplasia and eventually tumorigenesis.

2.4.2.2 Colon Cancer

It has become evident that the accurate coordination of both the Notch and the Wnt signals controls intestinal epithelial cell fate decisions and it is essential in normal intestinal development and consequently it may play an important role in intestinal tumorigenesis [119, 120]. Indeed, tracing cells in which Notch-1 was

activated, or detecting expression of the Notch target gene Hes-1 [3], indicated uniform Notch-1 activation in adenomas of adenomatous polyposis coli (APC) mice as well as in human colon cancer cell lines and primary human colon cancer tissue samples [121] implying that Notch and Wnt signaling are simultaneously active in the proliferating adenoma cells. Reedijk et al. provide correlative evidence of active Notch signaling in human adenocarcinomas [122]. Gene expression of both Jagged ligands, Notch-1, LFNG, and Hes-1 was detected by in situ hybridization and shown to be at comparable or greater levels than normally observed in cells of the crypt base. In another study performed by Fre et al. the interplay between the two signaling pathways was assessed in vivo by modulating Notch activity in mice carrying either a loss- or a gain-of-function mutation of Wnt signaling [123]. The proliferative effect of active Notch signaling has on early intestinal progenitors requires Wnt signaling, whereas its influence on intestinal differentiation appears Wnt independent. This synergy was also observed in human intestinal adenomas. The analysis of Hes-1 expression in human colon cancer samples showed that 12 out of 15 polyps of both sporadic and hereditary low-grade adenomas present strong nuclear Hes-1 expression, whereas Hes-1 is either not detected or expressed at low levels in human adenocarcinomas. Similarly, Hey1, HeyL, and the Notch ligands JAG-1 and JAG-2 were expressed at higher levels in human adenomas than carcinomas. These observations warrant the conclusion that elevated Notch signaling in benign adenomas may contribute to the initiation of colorectal cancer. On the other hand high Notch signals in adenomas could be interpreted to maintain a tumor suppressive function, whereas it seems to be dispensable at later stages of colon cancer development.

2.4.2.3 Pancreatic Cancer

There is increasing evidence that link Notch to the development and/or progression of pancreatic cancer. First, multiple Notch receptors, ligands, and downstream target genes have been shown to be expressed in early metaplastic lesions (known as pancreatic intraepithelial neoplasms, PanIN) as well as in pancreatic adenocarcinoma tissue of mice and humans [124, 125], indicating that Notch expression might be an early event in the development of pancreatic cancer. In addition, Notch could possibly cooperate with other key pathways in pancreatic tumorigenesis. For example, TGF-α-induced EGF receptor signaling is frequently found in pancreatic cancers and transgenic overexpression of TGF-α in mice also results in acinar to ductal metaplasia and correlated with increased Notch signaling [125]. The K-ras protooncogene is mutated in most pancreatic adenocarcinoma but also in early stage lesions indicating that activating K-ras mutations occur at an early stage during pancreatic carcinogenesis [126]. Thus, simultaneous expression of NICD with an oncogenic form of K-ras in either pancreatic progenitors or mature acinar cells resulted in the development of PanIN lesions at time points where expression of NICD or K-ras alone did not lead to such lesions. These results strongly suggest that Notch and K-ras synergize and can cooperate to initiate pancreatic carcinogenesis in animal models [127]. Finally, experimental data on murine pancreatic adenocarcinoma

strongly suggest that Notch signaling can also promote progression from PanIN lesion to pancreatic adenocarcinoma and therefore could represent a therapeutic target [128, 129].

Although these studies are very encouraging, many issues remain to be resolved. These include elucidation of the role Notch plays in pancreatic cancer and whether interference with Notch influences disease outcome, how do Notch receptors and ligands get re-expressed during the development of early PanIN lesions or during their progression to pancreatic is also unclear, as is the question of which Notch target genes are activated during this process and what is their role.

2.4.2.4 Medulloblastoma

Multiple signaling pathways, which are involved in regulating neural stem cells, are also aberrantly activated in medulloblastoma, such as the sonic Hedgehog and the Wnt pathways [130, 131]. Analysis of primary medulloblastoma tumor samples also revealed increased mRNA expression of Notch-2 but not Notch-1. In 15 % of the examined tumors increased Notch-2 expression levels correlated with Notch-2 gene amplification suggesting that it may play a more important role for this neoplasm compared to the other Notch receptor family members [132, 133]. Moreover, increased Hes-1 expression correlated with poor patient survival prognosis [134].

Additional evidence that Notch signaling is involved in medulloblastoma is derived from experiments trying to interfere with the Notch cascade. Pharmacological inhibition of Notch activation or using soluble Delta ligands or small interfering RNAs (siRNAs) approaches induced apoptosis and led to pronounced reduction of viable cells in medulloblastoma cell lines and/or primary explant cultures [134, 135]. Reciprocal gain-of-function studies overexpressing N2ICD promoted cell proliferation, and tumor growth in xenotransplantation experiments. Somewhat surprisingly, similar experiments using N1ICD resulted in growth inhibition [134], which suggests that both Notch receptor exhibit very distinct functions, in contradiction with the in vivo finding [135]. How the N2ICD growth promoting function differs from a N1ICD mediated growth inhibitory function is unknown and needs further investigation.

2.4.2.5 Skin Tumors

Recent studies suggest that activation of the Notch signaling pathway is important to preserve the melanocyte SC (MSC) and may also play a role in melanoma progression. Microarray profiling comparing the gene expression pattern of normal melanocytes to human melanomas revealed up-regulation of Notch receptors, ligands, and downstream target genes. Notch-2 and Hey1 mRNA were overexpressed in melanoma cells compared to nevi and normal melanocytes [136]. Furthermore, JAG-2 mRNA is upregulated in highly invasive melanoma cell lines [137]. Massi et al. have also found that the expression of Notch-1 and Notch-2, as well as Notch

ligands, was upregulated in human "dysplastic nevi" and melanomas as compared with common melanocytic nevi [138]. These results suggest that the activation of Notch may represent an early event in melanocytic tumor growth leading to the hypothesis that up-regulation of Notch signaling may sustain tumor progression. The oncogenic effect of Notch-1 on primary melanoma cells was mediated by β-catenin, which was upregulated following Notch-1 activation [9]. Moreover, the oncogenic effect of activated Notch-1 is at least partially mediated through regulation of the MAPK and PI3K-Akt pathways and hyperactivated PI3K-Akt signaling results in the upregulation of Notch-1 through NF-κβ activity [139, 140]. In addition, Notch-1 signaling enhances tumor cell adhesion and increases N-cadherin expression, a cell adhesion protein whose expression is highly correlated with melanoma progression and metastasis [140].

Pinnix et al. have recently provided good evidence that deregulation of Notch signaling activity plays a specific role in promoting a transformed phenotype in human melanocytes and has defined the importance of Notch signaling in human melanoma [141]. Through analysis of a large panel of cell lines and patient lesions they could show that Notch receptors 1, 2, and 4 are overexpressed particularly when compared against primary melanocytes or normal human skin and that ectopic N1ICD expression resulted in the loss of E-cadherin expression and up-regulation of MCAM, two well-characterized events in melanoma development. These findings suggest that Notch signaling plays a specific role in promoting the transformed phenotype in human melanocytes and acts as a driving force in melanocyte transformation. Nonetheless, genetic loss-of-function analyses in established melanoma models need to be performed in order to convincingly demonstrate that Notch activation is an obligate event necessary for melanoma development and/or tumor progression.

In non-melanoma skin tumors, data suggesting that Notch has tumor suppressive activities in the skin is mostly derived from genetic mouse studies and correlative expression studies of human skin lesions. Conditional inactivation of several signaling components of the Notch cascade including Notch-1, Notch-1-Notch-2-Notch-3 concomitantly, RBP-J, and Presinilin1 and 2 in mouse skin results in hyperproliferation of the skin, hair loss, and epidermal cyst formation within less than 4 weeks [142, 143]. Skin tumors in mice lacking Notch-1 develop only after a long latency period (approximately 12 months), but removal of additional Notch components accelerates time to tumor onset to as early as ~70 days in mice heterozygous for Notch-2 and lacking Notch-1 and Notch-3 [144]. The spontaneous tumors of these mice are papillomas with a subset progressing to heavily vascularized basal cell carcinoma-like tumors and a few squamous cell like tumors. The long latency of tumor onset in Notch-1 deficient mice suggest that loss of Notch-1 signaling on its own is not sufficient to develop skin tumors. During the latency period additional mutations are likely to accumulate thus sufficiently deregulating growth leading to tumor development [144]. In this scenario Notch would cooperate with additional oncogenic mutations and thereby contribute to tumor development.

The genetic mouse data seem to be consistent with observations in human skin cancer. Human basal cell carcinomas exhibit downregulated Notch-1, Notch-2,

and JAG-1 expression [53]. Moreover, reduced expression of Notch-1, Notch-2, and Hes-1 was shown in a panel of human oral and skin squamous cell carcinoma cell lines, as well as in surgically excised squamous cell carcinomas from patients. In addition, suppression of Notch signaling in primary human keratinocytes that express an activated form of the Ras gene is sufficient to cause aggressive squamous cell carcinomas in xenograft models [145], as seen with mouse keratinocytes [146]. Downregulation of Notch-1 expression in human skin tumors may be linked to compromised p53 function, a regulator of Notch-1 expression [147, 148]. Interestingly, a similar link between suppression of Notch and p53 activity was reported for Ewing's sarcoma [149], possibly indicating that such a mechanism could be conserved in tissues where Notch has growth suppressive functions.

Although the skin is clearly the best-studied organ system in which Notch exerts tumor suppressive properties, much more work needs to be done to map all the tissues where Notch loss may promote cancer. The prostate [157], small cell lung cancer [158] and hepatocellular carcinoma [154] are all locations where loss of Notch signaling may promote dysplasia.

2.5 Therapeutic Approaches to Modulating Notch Signaling

For targeting purposes, some features of the Notch pathway have unique relevance. First, the fact that the Notch pathway members has not enzymatic activity and signaling cascade triggered by Notch-ligand interactions does not include and enzymatic amplification step means that "signal intensity" can be modulated very precisely by cellular regulatory mechanisms. As a result, the downstream effects of Notch activation are exquisitely dose dependent [159]. This means that complete shutdown of the pathway may not always be necessary to achieve a therapeutic effect. A second important feature is that the intracellular half-life of the active form of Notch, NICD, is generally very short, in the order of minutes, as we referred above, though it may be longer in transformed cells [109]. The Notch signal is essentially a short pulse of gene regulation [159], which implies that sustained inhibition may not always be necessary and that intermittent inhibition may be successful. A third key feature is that the effects of Notch are remarkably context dependent. This means that Notch signals can be used for different purposes in different cell types and that systemic inhibition of Notch signaling is likely to have a multitude of effects in different cell types. Therefore, for therapeutic purposes it necessary to determine whether there is a level (or timing) of Notch inhibition that is sufficient to attain efficacy in disease control without causing intolerable adverse events (Fig. 2.1).

There is definitely a potential therapeutic benefit for targeting Notch in cancers including CSC depletion, tumor angiogenesis reduction, differentiation induction, and even cell death [59]. The efficacy of targeting Notch in cancer will vary within cancer types; even within the same cancer, targeting Notch may result in different effects on tumor subpopulations [11–13, 59]. Several inhibitory strategies are currently tested in preclinical setting as well as in clinical trials, including the following:

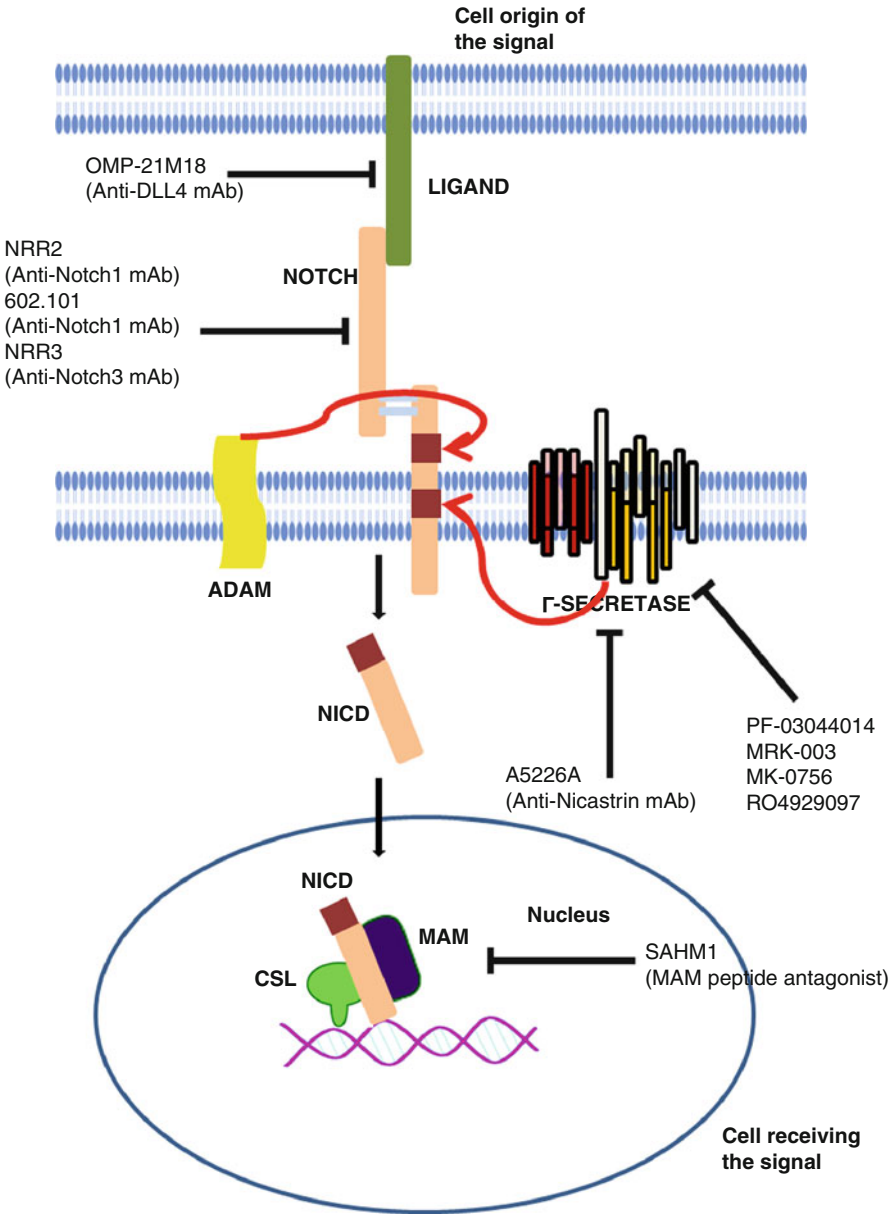

Fig. 2.1 Notch pathway. Alternative therapies to inhibit Notch pathway

1. Various GSI of different selectivity and efficacy, all preventing the S3 cleavage and thereby activation of all Notch receptors.
2. Inhibitory antibodies (Abs) against individual Notch receptors and ligands with the aim to block specific receptor-ligand interactions.

3. Receptor specific inhibitory Abs masking the S2 cleavage site, thereby blocking ADAM-protease-mediated cleavage of the receptors.
4. Novel stapled peptides blocking the formation of a functional NICD-MAML transcription complex of unknown selectivity and low efficacy.

Some on the more promising strategies, with their potential advantages and disadvantages, are discussed in detail below.

2.5.1 GSIs

GSIs were first developed as potential therapies to treat and/or prevent Alzheimer's disease, as the amyloid precursor protein (APP) implicated in this illness is also cleaved by the gamma-secretase [160, 161]. The off-target effects of the first-generation GSIs on Notch signaling immediately suggested that these agents might prove particularly useful in treating Notch-related tumors and they represent the pioneering class of Notch inhibitors both in the laboratory and in the clinic.

There are numerous GSIs (Table 2.1) commercially available for research and a number of chemical structures have been used as the basis for these compounds. The most commonly used is a modified di- or tri-peptide, usually with one to two aromatic hydrocarbon rings included. This has yield hydrophobic compounds which are cell-permeable and that act as reversible inhibitors of γ-secretase. In the laboratory, the most widely employed is DAPT and another frequently-used compound in the structurally similar Lilly GSI L685, 458. A structurally different compound which is also available preclinically is compound E (PF-03044014). Other classes of GSIs includes diazepine-type structures, with DBZ (dibenzazepine) as an example, agents based on an isocoumarin foundation, such as JLK6, that can bind and inhibit γ-secretase irreversibly or potent agents with a sulfonamide core, such as Compound 18 [64, 134, 172]. The specificity, selectivity, and dosing strategies of GSIs have been improving steadily over the last years. They have the advantage of relative ease of administration and oral bioavailability. In general, small molecules can be dosed more precisely than Abs because of their relatively short biological half-life and simpler dose-response relationships. An additional potential advantage is the fact that a single agent can block the activation of all four Notch homologues since they all depend on γ-secretase. Even though there are at least six different γ-secretase complexes in humans, and subtype-specific inhibitors might be developed, Notch appears to be a substrate for each of these complexes.

2.5.1.1 GSI for Notch-Targeted Cancer Therapeutics: T-ALL and Other Hematologic Malignancies

Given the well-documented role of overactive Notch signaling in T-ALL [6, 29, 89–99], many of the first studies to explore the potential efficacy of GSI-based cancer treatments focused on T-ALL human cell lines and mouse xenografts models.

Table 2.1 Preclinical and clinical trials evaluating γ-secretase inhibitors in cancer

Compound	Author, year	Status	Tumor/N° patients	Results
PF-03044014	Suwanjunee et al., 2008 [162]	Preclinical trial	Breast cancer xenograft model	In vivo: apoptosis, antiproliferation, reduced tumor cell self-renewal ability, impaired tumor vasculature and decreased metastasis activity. Significant antitumor activity in 10 breast xenograft models
	DeAngelo et al., 2006 [159]	Phase I	Refractory T-ALL/n = 10	1 PR Severe gastrointestinal toxicity
MRK-003	Tammam et al., 2009 [163]	Preclinical trial	Xenografts models of T-ALL	Notch signaling downregulation and induction of apoptosis. Complete tumour regression in mouse xenografts models of T-ALL
	Ramakrishnan et al., 2012 [164]	Preclinical trial	MM and NHL cell lines and patient cells	Treatment induced caspase-dependent apoptosis and inhibited proliferation of MM and NHL cell lines and patients cells
	Mizuma et al., 2012 [165]	Preclinical trial	Panel of human PDAC cell lines and patient-derived PDAC xenografts	MRK-003 monotherapy blocked tumor growth in 5 of 9 PDAC xenografts. MRK-003 + gemcitabine showed enhanced antitumor effects compared with gemcitabine in 4 of 9 (44 %) PDAC xenografts
MK-0752	DeAngelo et al., 2006 [159]	Phase I	Six adult and two pediatric patients with acute leukemia (7 T-ALL and 1 AML)	1 PR DLT was grade 3/4 diarrhea at 300 mg/m^2
	Krop et al., 2012 [166]	Phase I	Solid tumors refractory to standard therapy/n = 103	1 CR and 10 SD >4 months among patients with high-grade gliomas
	Fouladi et al., 2011 [167]	Phase I	Children with refractory or recurrent CNS tumors/n = 23	No objective responses. Prolonged SD (≥3 courses) in 2 patients

(continued)

Table 2.1 (continued)

Compound	Author, year	Status	Tumor/N° patients	Results
R04929097	Kolb et al., 2012 [168]	Preclinical trial	Childhood solid tumor and ALL xenografts	Significant differences in EFS distribution compared to control in 6 of 26 (23 %) of the evaluable solid tumor and in 0 of 8 (0 %) of the ALL xenografts
	Huynch et al., 2011 [169]	Preclinical	Human primary melanoma cell lines	Reduced proliferation and impaired ability to form colonies. Decreased tumor volume and blocked the invasive growth pattern of metastatic melanoma cells lines
	Tolcher et al., 2012 [170]	Phase I	Advanced solid tumors refractory to standard therapy/n = 110	2 PR, 1 minor response, 12 prolonged SD
	Strosberg et al., 2012 [171]	Phase I	Metastatic CRC patients who had received ≥2 lines CT/n = 37	No ORR, 6 SD Median PFS = 1.8 months Median OS = 6 months

T-ALL acute lymphocytic leukemia, *AML* acute myelogenous leukemia, *MM* multiple myeloma, *NHL* non Hodkin lymphoma, *PDAC* pancreatic adenocarcinoma, *CR* complete response, *PR* partial response, *SD* standard disease, *ORR* overall response rate, *PFS* progression free survival, *OS* overall survival, *EFS* event-free survival, *CNS* central nervous system, *DLT* dose limiting toxicity, *CT* chemotherapy

In an early study, five human T-ALL cell lines were found to undergo G_0/G_1 cell cycle arrest, reduction in cell proliferation, and increased apoptosis following treatment with Compound E [6], findings that were subsequently replicated with additional T-ALL cell lines [14, 15, 173–175]. Specific inhibition of Notch signaling was demonstrated by reduced NICD levels and transcriptional downregulation of Notch-1-responsive genes. Compound E was also shown to enhance the sensitivity of T-ALL cell lines to other agents, including dexamethasone and imatinib [14]. Similar effects on G_0/G_1 cell cycle arrest and apoptosis were also observed with the cyclic sulfonamide GSI MRK-003 for three T-ALL cell lines [176]. Nevertheless, these studies also revealed that only a subset of T-ALL cell lines responded positively to GSI therapy [6, 177] and mixed results were also reported with rodent xenografts models of T-ALL using various GSIs [15, 163]. For example, the GSI PF-03084014 was found to exert robust antitumor effects in six Notch-1-driven T-ALL xenografts [15], and MRK-003 similarly downregulated Notch signaling, inducing apoptosis and causing complete tumor regression in mouse xenografts of thirteen different human T-ALL lines [163]. However, the evaluation of the GSI RO4929097 in a panel of mouse xenografts representing several different human cancers revealed no effect on two T-ALL xenografts or six precursor-B ALL xenografts, although tumor growth delays were observed for other xenografts tested, particularly for osteosarcoma [168]. One likely factor that could explain these incongruent outcomes in T-ALL cell lines and xenograft models is that a variety of different GSI and dosing regimens have been employed in the studies performed to date, making it difficult to compare the results. Indeed, different T-ALL cell lines and xenografts are exquisitely sensitive to different dosing regimens. For instance, in one T-ALL xenograft study comparing seven different MRK-003 dosing regimens, high doses administered on a 3-days-on/4-days-off, weekly, or bimonthly schedule were effective, but moderately lower doses administered on similar schedules were ineffective in conferring antitumor protection [163]. In a second mouse xenograft study with PF-03084014, much stronger antitumor efficacy was observed for a 7-days-on/7-days-off dosing schedule compared to a 3-days-on/4-days-off dosing schedule [15]. These results highlight the necessity of carefully evaluating GSI dose levels, dosing schedules, and therapeutic windows to determine the optimal design of clinical trials for candidate GSI agents. A second factor contributing to the differential response of T-ALL tumors to GSI therapy is that these tumors are genetically heterogeneous. Several T-ALL cell lines that exhibit high levels of NICD are resistant to GSI treatment were found to harbor mutations in the FBW7 gene, which encodes an F-box ubiquitin ligase required for NICD degradation by the proteosome [177]. The FBW7 mutations abrogate its binding to the substrates, thus allowing NICD to evade its normal down-modulation. A significant percentage (8.1 %) of primary T-ALL isolates were also found to harbor FBW7 mutations, illustrating the tendency of T-ALL cells to acquire secondary mutations under selective pressure for continued tumor growth. Mutational inactivation of the PTEN tumor suppressor gene has also been documented in T-ALL cell lines with GSI resistance, resulting in hyperactive PI3K/Akt/mTOR signaling that confers this resistance by bypassing the requirements for Notch signaling during leukemic clone growth [71]. As these

studies indicate, it is of great importance to identify biomarkers and perform tumor genotyping in order to predict which molecular subtypes of T-ALL and other cancers are more likely to obtain benefit with GSI-based treatments.

A phase I clinical trial of MK-0752, which involved 10 patients with relapsed or refractory T-ALL, six of whom had activating Notch1 mutations, proved disappointing [159]. The orally administered daily dose (300 mg/m^2 per day) had sever gastrointestinal toxicities (diarrhea, fatigue and cough), and patients were on the trial for a median of 11 days. The dose and daily schedule were clearly too toxic. Evaluation of Notch1 levels in peripheral blood mononuclear cells did not correlate with gain-of-function Notch1 mutations or clinical benefit. Several phase I studies are currently underway to evaluate different GSI (MK-0752, RO4929097, MRK-003, PF-03084014) for the treatment of T-ALL.

On the other hand, emerging evidence indicates that GSIs might also prove useful for treating hematologic tumors of B-cell origin. Many B-cell tumor lines have been found to be sensitive to GSIs, providing a rationale for further testing in animal models and ultimately in human clinical trials. DAPT treatment causes a significant reduction in cell proliferation for Hodkin's lymphoma and multiple myeloma cells, and GSI-I, GSI-XII and DAPT inhibit growth and induce apoptosis of large B-cell lymphoma and Burkitt's lymphoma cells [173, 178]. Contradictory results have been obtained with precursor-B ALL cell lines, with one study reporting no significant effect of RO4929097 on six different cell lines [168], while another study found that GSI-I induces apoptosis in precursor-B ALL cell lines and primary lymphoblasts, and blocked or delayed engraftment in 50 % of precursor-B ALL mouse xenografts [179]. Furthermore, MRK003 treatment induced caspase-dependent apoptosis and inhibited proliferation of multiple myeloma and non-Hodkin lymphoma cell lines and patient cells. Examination of signaling events after treatment showed time-dependent decrease in levels of the notch intracellular domain, Hes1 and c-Myc. MRK003 downregulated cyclin D1, Bcl-Xl and Xiap levels in non-Hodkin lymphoma cells and p21, Bcl-2 and Bcl-Xl in multiple myeloma cells. In addition, MRK003 caused an upregulation of pAkt, indicating crosstalk with the PI3K/Akt pathway [164]. A complicating question is that unlike truly γ-secretases-specific GSIs, some GSI compounds including GSI-I and –XII also target the proteasome, leading to the suggestion that simultaneous inhibition of both γ-secretase and the proteosome is required for the pro-apoptotic activities of some GSIs in B-cell tumors [179]. Finally, GSI can also modulate the Notch-dependent interactions between B cells and stromal osteoblasts, osteoclasts and fibroblasts. GSI treatment ameliorates the stromal cell-mediated drug resistance of multiple myeloma cells in vitro and enhanced the antitumor activities of melphalan and doxorubicin in a murine multiple myeloma model [75]. Therefore, GSI-based therapies might prove broadly applicable for B-cell neoplasias despite the complexities of Notch signaling and its crosstalk with other pathways in this class of hematologic tumors.

2.5.1.2 GSI-Based Therapies for Solid Tumors

Numerous studies using human cancer cell lines and xenografts models have established the potential utility of GSI-based therapies for solid tumors [13, 64, 75, 83, 128, 162, 165, 168, 169, 180–183]. For instance, the in vitro and in vivo properties

of PF-03084014 have been investigated in a panel of breast cancer xenografts models [162]. In vitro, PF-03084014 exhibited activity against tumor cell migration, endothelial cell tube formation, and mammosphere formation. In vivo, apoptosis, anti-proliferation, reduced tumor cell self-renewal ability, impaired tumor vasculature, and decreased metastasis activity after the treatment of PF-03084014 was also observed. PF-03084014 treatment displayed significant antitumor activity in 10 of the 18 breast xenograft models [180]. However, the antitumor efficacy in most of them did not correlate with the in vitro antiproliferation results in the corresponding cell lines, suggesting the critical involvement of tumor microenvironment during Notch activation. In the tested breast xenograft models, the baseline expressions of the Notch receptors, ligands, and the cleaved Notch-1 failed to predict the antitumor response to PF-03084014, whereas several Notch pathway target genes, including Hey2, Hes-4, and Hes-3 were strong predictors of response. In addition, RO4929097 has shown to downregulated the Notch target genes Hes-1, Hey1, and HeyL in inflammatory breast cancer cells. However, the putative self-renewal mammosphere formation assay efficiency was increased with the drug [181]. Authors further showed that RO429097 inhibits normal T-cell synthesis of some inflammatory cytokines, including TNF-α and interleukin-8 (IL-8) production in the microenvironment. The therapeutic effect of GSI in K-rasG12V-driven non-small cell lung cancer (NSCLC) has also been shown in mice carrying autochthonous NSCLCs [182]. Treated carcinomas present reduced Hes-1 levels and reduced phosphorylated ERK without changes in phosphorylated MEK. Mechanistically, Hes-1 directly binds to and represses the promoter of DUSP1, encoding a dual phosphatase that is active against phospho-ERK. Accordingly, GSI treatment upregulates DUSP1 and decreases phospho-ERK. A recently published study has analyzed a panel of human pancreatic cancer cell lines as well as patient-derived pancreatic cancer xenografts to determine their responsiveness to MRK-003, a potent and selective GSI [165]. Pretreatment of pancreatic cancer cells with MRK-003 in cell culture significantly inhibited the subsequent engraftment in immunocompromised mice. MRK-003 monotherapy significantly blocked tumor growth in 5 of 9 (56 %) pancreatic cancer xenografts. A combination of MRK-003 and gemcitabine showed enhanced antitumor effects compared with gemcitabine in 4 of 9 (44 %) pancreatic adenocarcinoma xenografts, reduced tumor cell proliferation, and induced both apoptosis and intratumoral necrosis. Gene expression analysis of untreated tumors indicated that upregulation of NF-κB pathway components was predictive of sensitivity to MRK-003, whereas upregulation in B-cell receptor signaling and nuclear factor erythroid-derived 2-like 2 pathway correlated with response to the combination of MRK-003 with gemcitabine. Finally, RO4929097 reduces the tumor initiating potential of human melanoma cell lines by decreasing the levels of Notch transcriptional target Hes-1. RO4929097 also decreased tumor volume and blocked the invasive growth pattern of metastatic melanoma cell lines in vivo [169]. Another GSI, MRK-003 has also shown to reduce growth and cell invasion in uveal melanoma cell lines [183].

Several phase I clinical trials with GSIs have also been performed in patients with solid tumors [184–187]. The primary aims of these studies were to determine a maximum-tolerated dose (MTD) for phase II dosing, assess safety, and examine

potential antitumor efficacy, as well as pharmacokinetic (PK) and pharmacodynamic (PD) end points. In the study by Krop et al. [166], MK-0752 was re-investigated in 103 patients under three schedules and at flat doses: schedule A with continuous, once-per-day dosing; schedule B with dosing on 3 days of 7; and schedule C with once-per-week dosing. Both schedules A and B evaluated two cohorts before dose-limiting diarrhea occurred with schedule A and unexpected dose-limiting fatigue occurred with schedule B. Dose expansions at the lower dose of 450 mg on both schedules indicated unacceptable toxicity; therefore, a weekly dosing schedule was pursued. However, by the time it was decided that neither schedule A nor B was worth pursuing, a total of 38 patients had already been treated in the expansion cohort. It is unclear why schedule A or B was re-investigated when the dose and the schedules were clearly too toxic in the previously reported MK-0752 study in patients with T-ALL. Not surprisingly, identical toxicities were reported in both studies. On schedule C (once-per-week dosing), 65 patients were treated in eight cohorts. Significant inhibition of Notch signaling was observed with the 1,800–4,200-mg weekly dose levels, confirming target engagement at those doses. Clinical benefit was observed, with one objective complete response and an additional 10 patients with stable disease longer than 4 months were observed among patients with high-grade gliomas. Although this drug has been reported to penetrate the CNS in mouse models, how effectively this occurs in patients is unknown. A second study with MK-0752 has been performed in 23 children with refractory or recurrent CNS malignancies [167]. MK-0752 was administered once daily for 3 consecutive days of every 7 days at escalating dosages starting at 200 mg/m^2. Interestingly, no clinical responses or durable stable disease has been reported. Perhaps, this reflects lower daily dosing and generally low plasma levels, below which CNS penetration was observed in the animal models.

Tolcher et al. have evaluated three schedules of oral RO4929097 in 110 patients with refractory locally advanced or metastatic solid tumors [170]. In schedule A, 58 patients were treated for 3 days on and 4 days off for the first 2 weeks, followed by a week of rest. In schedule B, 47 patients were treated for the first 7 consecutive days of a 3-week cycle. Despite multiple dose escalations, an MTD (using a classic 3 +3 design) could not be defined for either schedule A or B. In fact, dose escalation was halted at a dose of 270 mg on schedule A and 135 mg on schedule B because PK evidence indicated CYP3A4 autoinduction at doses greater than 24 mg on schedule A and 18 mg on schedule B, with a decline in plasma concentrations with continued dosing. Treatment was well tolerated at the doses of both schedules A and B. Tumor responses included one partial response in a patient with colorectal adenocarcinoma with neuroendocrine features, one mixed response (stable disease) in a patient with sarcoma, one nearly complete FDG-PET response in a patient with melanoma and prolonged stable disease in several other tumor types.

The activity of RO4929097 has recently been tested in a phase II trial including 37 patients with metastatic colorectal cancer who had received at least two prior lines of systemic CT. Patients were treated at the dose of 20 mg daily, 3 days on and 4 days off continuously. No objective radiographic responses were observed and only six patients had stable disease as their best response. Median progression

free survival (PFS) was 1.8 months and median overall survival (OS) was 6 months, which suggests that RO4929097 at the study dose has minimal single agent activity in this malignancy [171].

GSI-based Notch inhibition is now being evaluated for some solid tumors in preclinical and clinical trials that are currently underway. In fact, the Cancer Therapy Evaluation Program now has several trials that are readdressing the issue of optimal dose and schedule (A Phase I Study of Various Administration Schedules of RO4929097 With Multi-Parameter Assessment [Biomarkers, Pharmacokinetics, Pharmacodynamics] in Patients With Advanced Solid Cancers; and the Randomized Drug Interaction Study of RO4929097 for Advanced Solid Tumors). Several studies are in fact using schedules not even tested in phase I studies, such as daily low doses of drugs (15 mg per day) so as to avoid autoinduction (Phase IB/II Study of GDC-0449 [NSC 747691] in Combination With RO4929097, a Gamma-Secretase Inhibitor [GSI] in Advanced/Metastatic Sarcomas). All of these issues might have been avoided by examining the tumors of the patients. It is thus quite clear that it is essential to examine pharmacology in real time. As newer biologic and small molecule inhibitors are investigated, we will continue to encounter agents for which an MTD cannot be readily defined. In this context, pathway inhibition in matched-pair tumor samples will be absolutely critical to help define the minimal biologically effective dose and optimize the drug development process [188].

Preclinical and clinical trials evaluating GSIs in cancer are summarized in Table 2.1. Table 2.3 showed ongoing clinical trials with these compounds.

2.5.2 Notch Immunotherapy: Antibody Inhibitors of Notch Activity

An alternative approach for inhibiting Notch signaling is immunotherapy using antibodies directed against Notch, its Delta/Jagged ligands, or other components of the pathway (Table 2.2). One potential advantage of Abs inhibitors is their specificity, allowing specific members of the pathway to be targeted with high affinity, potentially limiting mechanism-based toxicity caused by global inhibition of Notch signaling [11–13]. One situation in which a specific biologic may be preferable is in malignancies where a particular mutated or otherwise deregulated Notch paralogue is known to be the primary oncogenic event or if the target has a relatively restricted expression pattern compared to other pathway members. Abs, however, are large molecules, though the delivery/access to cancer cells could be a main difficulty. For certain cancers such as brain tumors, local delivery may be an option, but for most metastatic cancers it is necessary to have efficient systemic distribution. Thus, inhibitory Abs of Notch may be most easily applied toward hematopoietic malignancies or for antiangiogenic purposes. Other potential disadvantages of biologics in this setting include their generally complex dose-response curves in vivo and their long biological half-lives. If intermittent inhibition of Notch signaling is desirable to minimize adverse events, using an mAb that will remain in circulation

Table 2.2 Preclinical trials evaluating antibodies inhibitors of Notch activity and peptide-based approaches

Compound	Author, year	Status	Tumor	Results
OMP-21M18 (Anti-DLL4 mAb)	Hoey et al., 2009 [189]	Preclinical trial	Xenograft models derived from primary human tumors	Tumor growth inhibition through multiple mechanisms, including a reduction in CSC frequency and a disruption of productive angiogenesis
	Liu et al., 2011 [190]	Preclinical trial	Xenograft models derived from human colorectal carcinoma and head and neck cancer	The combination of anti-DLL4 mAbs and ionizing radiation impairs tumor growth by promoting nonfunctional angiogenesis and tumor necrosis
	Fischer et al., 2011 [191]	Preclinical trial	Human colorectal tumor xenograft models	Anti-DLL4 was efficacious against WT and mutant KRAS colon tumors as a single agent and in combination with irinotecan
NRR2 (Anti-Notch1 mAb)	Wu et al., 2010 [186]	Preclinical trial	Xenograft models derived from human T-ALL	Inhibition of Notch 1 and Notch2 causes severe intestinal toxicity, the inhibition of either receptor alone reduces this effect
602.101 (Anti-Notch1 mAb)	Sharma et al., 2012 [192]	Preclinical trial	Breast cancer cell line MDA-MB-231	Inhibition of ligand-dependent expression of downstream target genes of Notch, cell proliferation and induction of apoptotic cell death
NRR3 (Anti-Notch3 mAb)	Li et al., 2008 [185]	Preclinical trial	Human T-ALL cell lines	The inhibitory Notch3 Abs reduces cellular proliferation, survival and motility. Activating Abs mimics the effects of ligand-induced Notch activation
A5226A (Anti-Nicastrin mAb)	Hayashi et al., 2012 [193]	Preclinical trial	T-ALL cell T-ALL mouse xenografts	Inhibition of the γ-secretase activity-dependent growth of cancer cells
SAHM1 (MAM peptide antagonist)	Moellering et al., 2009 [194]	Preclinical trial	Murine and human T-ALL cell lines T-ALL mouse xenografts	SAHM1, prevented MAML1 from binding to the NICD-CSL complex and blocked the expression of Notch-1 target genes, and reduced proliferation of T-ALL cell lines

T-ALL acute lymphocytic leukemia, *DLL4* delta-like ligand 4, *CSC* cancer stem cells, *mAb* monoclonal antibody, *WT* wild-type, *NICD* Notch intracellular domain, *NRR* negative regulatory region

for days or weeks may prove challenging in terms of regimen designs. Of course, the biological half-lives of mAbs can be modulated by recombinant engineering or generation of F(ab)2 s, F(ab)s or even single chain Fvs [11, 13].

Inhibitory Abs directed against Notch ligands, including DLL-1 and DLL-4 have been developed. As mentioned above, Notch signaling via the ligand DLL-4 was reported by multiple groups to suppress angiogenic sprouting by endothelial cells and DLL-4 overexpression is found in tumor vasculature and in tumor cells to activate Notch signaling [79, 80]. Studies targeting blood vessel formation employing blocking Abs to DLL-4 revealed substantial tumor growth reduction in cancer cell line-based xenograft models [80]. The antitumor effect was shown to be the result of deregulated angiogenesis characterized by increasing sprouting in endothelial tip cells leading to chaotic and dysfunctional vasculature in the tumor. Thus, inhibiting DLL-4 disrupts productive angiogenesis in a different way from traditional antiangiogenic therapies causing hyperproliferation of tumor vessels that leads to a reduction in tumor growth. Importantly, this occurred even in cancer models that were resistant to VEGF Abs, an established and powerful antiangiogenic approach in cancer therapy [79, 80, 195–197]. This has prompted an aggressive effort to develop DDL-4 Abs for clinical usage. A land mark study by Hoey and colleagues demonstrated that blocking DLL-4 signaling inhibits tumor growth through multiple mechanisms, including a reduction in CSC frequency. In addition to the previously described effect on deregulating angiogenesis, they showed that selectively inhibiting DLL-4 signaling in human tumor cells with a humanized anti-hDLL4 21M18 Ab leads to a decrease in colon tumor growth, a delay in tumor recurrence after chemotherapeutic treatment, and a decrease in the percentage of tumorigenic cells. In a second study, the combination of specific DLL-4 Notch blockade and ionizing radiation impairs tumor growth in human colorectal carcinoma and human head and neck xenografts by promoting non-functional tumor angiogenesis and extensive tumor necrosis, independent of tumor DLL-4 expression [198]. Fischer et al. tested the efficacy of anti-DLL4 antibodies in KRAS mutant tumors in a panel of early passage colon tumor xenograft models derived from patients. It was efficacious against both wild-type and mutant KRAS colon tumors as a single agent and in combination with irinotecan [199]. Further analysis of mutant KRAS tumors indicated that the anti-DLL4/irinotecan combination produced a significant decrease in colon cancer stem cell frequency while promoting apoptosis in tumor cells. Following those preclinical studies providing evidence of antitumor activity, phase I clinical trials of the use of two different anti-DLL4 human mAbs- REGN421 and OMP-21M18- in treatment of solid tumors are currently in progress. A recent report, however, has raised some important safety concerns in the use of blocking DLL-4 chronically [200]. The authors showed that prolonged DLL-4 blockade using a rat model resulted in severe disruption of normal tissue homeostasis, caused pathological activation of endothelial cells and ultimately led to the development of vascular/endothelial cell-based tumors resembling hemangioblastoma in skin, heart, and lung. If this adverse event is borne out by others, it may present a major obstacle to the usage of DLL-4 Abs in clinic.

Blocking Abs to Notch or its ligands may serve not only antiangiogenic functions but also directly inhibit cancer cells. A growing number of reports have described the development of Abs that specifically antagonize the Notch paralogues Notch-1, 2 and 3. Some of these Abs seem to work by recognizing and stabilizing the extracellular NRR of Notch that undergoes a conformational change upon ligand bindings to facilitate ADAM protease cleavage at the S2 site [201–204]. This raises the interesting prospect that Abs could fine-tune Notch activity, increasing or attenuating signaling by individual Notch family members by different mechanisms. Several in vitro studies on human tumor lines indicate that they are able to inhibit oncogenic Notch signaling, albeit not as potently as cell-penetrating, small molecule GSIs [203]. One interesting report has emerged in which anti-NRR Abs were developed that specifically block activity of either Notch-1 or Notch-2 [202]. The Notch-1 anti-NRR showed good antitumor effects, but without the gut toxicity associated with combined Notch-1 and Notch-2 inhibition. Sharma et al. have developed the mAb 602.101, which specifically recognizes Notch-1, inhibited ligand-dependent expression of downstream target genes of Notch such as Hes-1, Hes-5, and HEY-L in the breast cancer cell line MDA-MB-231 [204]. The mAb also decreased cell proliferation and induced apoptotic cell death. Furthermore, exposure to this Ab reduced CD44(Hi)/CD24(Low) subpopulation in MDA-MB-231 cells, suggesting a decrease in the cancer stem-like cell subpopulation. This was confirmed by showing that exposure to the Ab decreased the primary, secondary, and tertiary mammosphere formation efficiency of the cells. The Ab also modulated expression of genes associated with stemness and epithelial-mesenchymal transition. In a third study, Abs to Notch-3 were also reported that can either block or stimulate receptor signaling [201]. These findings provide insights into the mechanisms of Notch autoinhibition and activation and pave the way for the further development of specific antibody-based modulators of the Notch receptors, which are likely to be of utility in a wide range of experimental and therapeutic settings. A number of Notch-targeting Abs (NNR-1, NNR-1, NNR-3) are currently being evaluated in preclinical studies, and the anti-Notch mAb OMP-59R5 is under phase I clinical trial evaluation (Table 2.3).

MAbs could also be used to target γ-secretase itself, an approach that has received little attention due to the availability of highly effective GSIs. Nevertheless, human γ-secretase is heterogeneous, with at least six different complexes possible due to differential usage of either presenilin-1 or –2 as well as Aph-1Aς, Aph-$1A_L$ or Aph-1B, and targeted inhibition of particular γ-secretase subtypes could potentially be beneficial in some therapeutic contexts. In a recent published study, a novel mAb A5226A against the extracellular domain component Nicastrin has been shown to inhibit γ-secretase activity by competing with substrate binding, and to interfere with proliferation of T-ALL cell lines and tumor growth in T-ALL mouse xenografts [42].

Preclinical trials evaluating antibodies inhibitors of Notch activity are summarized on Table 2.2.

Table 2.3 Ongoing clinical trials evaluating therapeutic targeting of Notch signalling

Compound		Condition	Status
γ-secretase inhibitors	MK-0752	CNS tumors Breast cancer Pancreatic cancer T-ALL	Phase I clinical trials: NCT00756717 NCT00803894 NCT01295632 NCT01098344 NCT00645333 NCT01243762 NCT00572182 NCT00106145 NCT00100152
	RO4929097	Breast cancer CNS tumors Colorectal cancer Melanoma T-ALL	Phase I clinical trials: NCT01198535 NCT01218620 NCT01217411 NCT01149356 NCT01270438 NCT01238133 NCT01088763 NCT01141569 NCT01208441 NCT01196416
	PF-03084014	Solid tumors T-ALL	Phase I clinical trial: NCT00878189
MABs targeting notch signaling	OMP-21M18 (Anti-DLL4mAb)	Pancreatic cancer Colorectal cancer Non-squamous non-small cell lung cancer Solid tumors	Phase I clinical trials: NCT01189929 NCT01189942 NC01189968 NC00744562
	OMP-59R5 (Anti-Notch mAb)	Pancreatic cancer Solid tumors	Phase Ib/II clinical trial NCT01647828 NCT01277146

2.5.3 Peptide-Based Approaches

The transcriptional effector complex downstream of Notch has also been targeted using a different-based approach. A recent publication has converted the peptide MAML1 transcription factor-based inhibitors into a drug-like molecule able to target the Notch/CSL transcription complex [194]. The crystal structure of Notch/MAML1/CSL identifies a nearly continuous stretch of α-helices at the interface of the three proteins. Moellering et al. hypothesized that a helical peptide mimetic might be able to compete for binding to NICD with full-length MAML1 and therefore inhibit transcriptional activation of Notch-targeted genes [205]. The researchers designed a series of six stapled α-helices peptides derived from MAML1, thus named for covalent backbone bonds stabilizing the helix. The stapled peptide was

also more resistant to protease recognition and degradation and it was actively taken up by cells and entered the nucleus, where they can target the transcriptional process. In vitro cell culture studies confirmed that one peptide, SAHM1, prevented MAML1 from binding to the NICD-CSL complex, blocked the expression of Notch-1 target genes, and reduced proliferation of human T-ALL cell lines. The inhibitory effect was confirmed in a murine model of T-ALL reducing tumor burden significantly compared with vehicle. This strategy holds promise and, in principle, it could be applied to other components of the Notch pathway, including ADAM10/17 proteases or Notch glycosylation enzymes, although an important consideration in the extent to which such agents might also disrupt other cellular processes that depend upon the same enzymes.

2.5.4 Combinatorial Therapies Involving Notch Inhibition

As is becoming clear for many targeted inhibitors in cancer, Notch inhibition may be best not as solitary therapy but in combination with other agents. Such combinations will be made possible only through a thorough understanding of cross-talk between Notch and other developmental and non-developmental pathways that may play roles in specific malignancies. Given that deregulated Notch signaling plays an ancillary role in many cancers that are primarily caused by malfunction of other signaling pathways and cell growth mechanisms and the growing body of evidence demonstrating that Notch inhibitors sensitize to more standard treatments such as chemotherapy and radiotherapy [206–208], a promising approach is to combine Notch inhibition with other chemotherapeutic agents that target these other pathways. In T-ALL cell lines harboring both Notch-1 mutations and Abl1 fusions, certain combinatorial treatment regimens using GSIs with the kinase inhibitor imatinib have demonstrated synergistic antitumor effects [14]. Real et al. achieved a promising breakthrough using the combination of a GSI with dexametasone in glucocorticoid-resistant tumor cell lines [103]. Moreover, dexamethasone counteracts lethal gut toxicity induced by the GSI and the authors outline how the combination therapy induces apoptosis in T-ALL cell lines, primary human T-ALL cells, and in xenografts of such T-ALL cell lines in mice to a much greater extent than either dexamethasone or the GSI alone. A second study has shown similar conclusions. Combination treatment of the GSI PF-03084014 with glucocorticoids induced a synergistic antileukemic effect in human T-ALL cell lines and primary human T-ALL patient samples. Mechanistically, PF-03084014 plus glucocorticoid treatment induced increased transcriptional upregulation of the glucocorticoid receptor and glucocorticoid target genes. Glucocorticoid treatment effectively reversed PF-03084014-induced gastrointestinal toxicity via inhibition of goblet cell metaplasia [209]. Synergistic effects were not observed, however, when GSIs were combined with etoposide, methotrexate, vincistine or L-asparaginase [14]. In multiple

myeloma, combined inhibition of Notch using GSI-XII treatment and Bcl-2/Bcl-xL using the small molecule ABT-737 resulted in synergistic cytotoxicity in cell lines and mouse xenografts models [209]. Enhanced antimyeloma effects have also been observed for combinations involving GSI-XII and bortezomib in cell lines and primary bone marrow isolates [210].

The utility of combining GSIs with conventional chemotherapy, hormonotherapy or targeted agents has also been confirmed for solid tumors [211–219]. In breast cancer, GSIs such as LY-411,575 and MRK-003 were found to prevent or reduce ErbB-2-positive tumors recurrence when combined with lapatinib or trastuzumab in cancer xenografts, and partially reversed trastuzumab resistance in refractory tumors [211]. Notch signaling is prominently regulated by Her2/Neu and trastuzumab-induced inhibition of ErbB-2 leads to Notch-1 activation [212], which in turns activates PI3K/Akt/mTOR signaling [70, 213], a tumor-promoting event that is attenuated by GSI treatment in some ErbB-2-positive breast cancer lines [69, 212]. In addition, a newly discovered feedback between Notch and the ER-α [112, 137, 214] supports combining Notch inhibitors with anti-estrogens and this combination is being investigated in ongoing clinical trials. An unexpected but potentially useful observation was that co-treatment with tamoxifen greatly alleviated the intestinal toxicity of orally administered GSIs, suggesting that this combination may be not only more effective but also safer than single GSI treatment. Oxaliplatin-induced activation of Notch-1 signaling in metastatic colorectal cancer is reduced by simultaneous GSI treatment, resulting in enhanced tumor sensitivity to oxaliplatin [215]. Additionally, the combination of PF-03084014 and irinotecan may be effective in reducing tumor recurrence in a colorectal cancer preclinical explants model in those tumors exhibiting elevated levels of the Notch pathway [216]. Synergistic anti-tumor effects have also been documented recently for the GSI MRK-003 together with rapamycin in pancreatic cancer, which was attributed to enhanced inhibition of the PI3K/Akt/mTOR pathway by the combined therapy [217].

Inhibitors of the PI3K-AKT-mTOR pathway may also be useful in combination with Notch inhibitors, and there is evidence that this strategy may reverse resistance to GSI in T-ALL that carry PTEN inactivating mutations [71]. Whether this strategy can be successful in other cancers characterized by PTEN loss is unclear. The complex cross talk between Notch and NF-κB suggests that, at least in some circumstances, drugs that inhibit NF-κB activity directly or indirectly could be successfully combined with Notch inhibitors [40, 218, 219]. As DLL-4 mAb appear to be effective independently of VEGF, they may be useful in combination with agents that block the VEGF pathway such as bevacizumab [78–81]. Finally, a particularly interesting treatment option is the combination of Notch inhibitors with inhibitors of other key stem cell pathways. For example, recent results show potent anti-cancer effects from combining a Notch-inhibiting agent and a Hedgehog pathway inhibitor in glioblastoma stem cell lines [220]. Hedgehog-inhibitor plus GSI combinations are also being investigated in ongoing clinical trials in breast cancer.

2.5.5 Future Approaches for Notch Inhibition

2.5.5.1 Other Potential Approaches to Small-Molecule Inhibitors

At the theoretical level, it could be possible to generate small-molecule inhibitors of Notch that act at different levels. Whereas attention has focused only on γ-secretase as a vulnerable point in Notch processing, it may also be feasible to use α-secretase inhibitors (ASI). The α-secretase enzymes that cleave Notch are thought to be ADAM-7 and ADAM-10 [221], and inhibitors that block both these ADAMs have been developed [222]. There may be theoretical advantages of an ASI over a GSI; for example, an ASI would not have to enter the cell to act. The process of testing ASIs as Notch inhibitors in cancer is currently underway.

While the inhibition of an enzymatic activity is typically the most common strategy to block a protein or pathway, examples are beginning to emerge of the potential druggability of protein-protein interactions. This strategy has been shown in reports in which small-molecule agents were derived to interrupt the interaction of the fusion protein EWS-Fli 1 with the RNA helicase RHA [223] or the p53/MDM2 interaction [224]. A number of protein-protein interactions in the Notch pathway would be logical targets for disruption, including Notch-Notch ligand, NICD-CBF1 transcription factor, or NICD-MAML.

Another promising approach relies on the discovery that the γ-secretase cleavage of Notch occurs not at the cell membrane but in the acidic endosomes [225]. A number of agents with the potential to interfere with endosomal acidification have been screened for their ability to reduce Notch activity. The Na+/H+ antiporter Monensin emerged as a potent Notch inhibitor [226].

2.5.5.2 Genetic Strategies

Genetic strategies for Notch inhibition may also find limited application in cancer therapy, particularly for hematopoietic malignancies or localized tumors, such as in lung or brain. One potential option could consist of delivery of a gene or pseudogene encoding a Notch-inhibiting peptide or protein. A dominant-negative form of MAML has been used in vitro to inhibit canonical Notch signaling via CBF1 [227] and other genes known to down-regulate Notch could also serve this function, such as the Numb/Numb-like or FBXW-7 genes [228]. Agents that up-regulate the expression of these endogenous Notch-inhibiting genes could be another way of blocking Notch activity.

Delivery of RNA interference represents a similar strategy for Notch-inhibiting cancer therapy, but possibly one with more potential for clinical success. Similarly to Notch-inhibiting genes, delivery remains one of the main problems in developing such strategies, but it is relatively less challenging to deliver small oligonucleotides than its whole genes. Either siRNAs or endogenous or artificial microRNAs could be generated. Each microRNA targets numerous genes and in general each gene is targeted by more than one microRNA. MicroRNAs thus offer the potential to simultaneously target more than one gene of interest, though the target genes

may not be suppressed as efficiently as by siRNAs. For example, the microRNA miR-326 has been shown to target both Notch-1 and Notch-2 and to decrease Notch activity [229]. The tumor-suppressive micro-RNA miR-34a has also been shown to target Notch-1 and Notch-2 and microRNA-206 has targeted Notch-3. In some cases, transfecting these microRNAs has demonstrated not only to decrease Notch activity but also to kill cancer cells, as in the case of the miR-326 and glioblastoma cells [229]. In addition, viral or liposomal vectors have been developed for genetic strategies such as RNA interference. Recent studies suggest that cancer cells have been shown to shed large amounts of microvesicles that can transmit cytoplasmic contents to nearby cells and that siRNAs or microRNAs can be transferred in this fashion to suppress gene expression in those cells. Thus, even if a limited percentage of cancer cells is transfected with a therapeutic vector, the transfected cancer cells may "share" with neighboring untransfected cancer cells to obtain benefit. It remains an open question, however, whether siRNAs or microRNAs would be successful agents for Notch inhibition and cancer therapy.

2.5.6 Potential Risks of Notch Inhibition

Different strategies for Notch inhibition in cancer may also pose potential significant risk and side effects. As with the initial evaluations of GSIs for the treatment of Alzheimer's disease, studies involving first-generation GSIs in T-ALL and other tumors found that this therapy fails to distinguish individual Notch receptors, inhibits other signaling pathways and cause significant systemic toxicity, attributed to dual inhibition of Notch-1 and 2 [203]. One of the most common adverse events is severe diarrhea. This is likely an on-target side effect of Notch inhibition, given that Notch drives gastrointestinal precursor cells toward an epithelial fate and away from a secretory cell fate; therefore, chronic Notch inhibition in the gut causes metaplastic conversion of proliferative cells in intestinal crypts and adenomas into secretory globet cells [3, 5, 46]. Secretory diarrhea showed its potential to be a dose-limiting toxicity in all these trials and it is likely one that will be problematic for any systematically-delivered Notch inhibitor. However, it has been found that intermittent rather than continuous oral administration of GSIs (e.g. giving a week off each month) greatly ameliorates the intestinal toxicity, presumably because it allows at least some intestinal stem cells to correctly differentiate as enterocytes. Corticosteroid treatment may also help to minimize the gut toxicity of Notch inhibition while maintaining anti-tumor efficacy [103]. Other adverse effects of systemic GSI treatment in mice include reversible thymic suppression, reversible hair depigmentation, hair loss or reversible hyperkeratosis. Hair loss in dose-escalation experiments is an indication that a toxic dose has been reached and is associated with diarrhea and weight loss. GSI are not significantly myelotoxic, making such a potential application at least theoretically feasible.

There has also been concern about other two theoretical risks of long-term Notch inhibition have been posited. The first one is the potential for damage or ablate

the normal adult stem cell populations in various organs, which possible impact is difficult to determine, but could include anything from hematopoietic collapse to subtle cognitive dysfunction. No signs of such toxicities have been uncovered in the earliest clinical trials, but the dosing was relatively short in those studies. The second potential risk may be even more concerning, as it involves an increased incidence of certain cancers. Whereas Notch plays an oncogenic role in most tissues, it acts as a tumor suppressor in some, such as B lymphocytes, neuroendocrine lung cells and certain skin cells [146, 158]. For example, loss of Notch signaling in the skin causes a barrier defect that causes local inflammation, predisposing to transformation, hyperproduction of thymic stromal lymphopoietin, and systemic immunological disturbances [142, 143]. Thus long-term Notch inhibition may increase the risk of cancers in these cellular compartments, though this has not yet been demonstrated.

2.6 Conclusions and Future Perspectives

Notch is a critical pathway in stem cell maintenance, development and cancer [2–5]. It has been shown to be important in numerous hematologic and solid malignancies [6–10, 105–156], and its potential utility against "tumor stem cells" makes it a particularly high-value target [59, 63–65]. Despite last years have seen rapid advances in the development of therapeutic approaches to treat cancers and other diseases associated with dysfunctional Notch signaling, patients are not yet routinely treated by deliberately targeting the Notch pathway aside from clinical trials. Most Notch-directed therapies involve the use of GSIs, that have long been used in basic and preclinical investigation [13–15, 64, 75, 83, 128, 162, 165, 168, 169, 180–183] as Notch inhibitors and they have recently begun clinical trials in cancer patients [166, 167, 170, 171, 184–191, 195–197] (Table 2.3). Other more selective inhibitors of Notch and Notch ligands, such as DLL-4 Abs, are in preclinical or early clinical development and may show great promise against not only cancer cells but also tumor angiogenesis [79, 80, 184–187, 189–197]. Optimism for Notch should be tempered somewhat by adverse events such as gastrointestinal toxicity that are beginning to be observed in clinical trials and other problems from long-term Notch inhibition remain to be discovered [142, 143, 145, 157, 184, 186, 223, 224]. Promising results have also been obtained through less common approaches, such as using Notch 1 ectodomain expression to inhibit tumor growth and angiogenesis [226], inhibiting the ADAM metalloproteases that perform key activating Notch cleavages [227], expressing dominant-negative fragments of MAML [215, 228] and expressing DLL-1 and JAG-1 fusion proteins to modulate Notch signaling [229]. Although all these strategies show great potential for realistic therapeutic intervention of Notch signaling in the future, they also highlight the need to identify groups of patients and/or subtypes of cancers who are most likely to benefit from Notch inhibitors. To that end, it remains to be determined which cancers and specific subtypes are characterized by active Notch signaling, what specific roles are performed by different components of the Notch signaling pathway in a given

tumor, and whether global or selective Notch modulation is most desirable. Another key point is the study of biomarkers on Notch-associated cancers to understand other cellular events and signaling pathways interactions that contribute to tumor progression, thereby guiding the selection of the most effective treatment, which in many cases will involve GSIs or Notch immunotherapy in combination with other chemotherapeutic or targeted agents.

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Chapter 3
Hedhehog as a New Paradigm in Cancer Treatment

Laura Visa, Cristina Nadal, and Pere Gascon

Abstract The Hedgehog (Hh) signalling pathway plays an important role in the formation and maintenance of cancer stem cell (CSC) and in the acquisition of epithelial-mesenchymal transition (EMT). Since these two properties are very relevant in cancer biology: cell invasion, metastasis, drug resistance and, the appearance of cancer relapse, the Hh pathway is considered an important target for future cancer treatments. Over the last few years, several small-molecules inhibitors have been designed and introduced in cancer clinical trials with some of them showing already very promising results. Currently, many of such inhibitors are in clinical development being tested in ongoing clinical trials. In addition, many products of the so called nutraceutical family (curcumin, soy isoflavones, vitamin D, resveratrol and epigallocatechin-3 gallate) have been shown to inhibit tumor growth through downregulation of the Hh signaling pathway. The inhibition of the Hh signalling pathway should led to the suppression of cancer cell growth, invasiveness, metastasis and eventually prevent tumor recurrences. The future design of novel strategies combining inhibitors of the Hh pathway with nutraceuticals and inhibitors of other signaling pathways to regulate activated Hedgehog could bring new tools for cancer treatment.

Keywords Hedhehog • Patched • Smoothened • Stem cells • Vismodegib

3.1 Introduction

It is well known that MAPK, Akt, NF-kB, Wnt, Hedgehog, Notch, estrogen and androgen receptors (ER) (AR), signalling pathways are essential pathways in tumor development and progression.

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The Hedgehog (Hh) family of proteins plays an important role in the regulation of cell growth, differentiation and cell survival. The Hh family participates in the formation and maintenance of cancer stem cell (CSC) and in the acquisition of epithelial-mesenchymal transition (EMT) [1, 2]. It also participates in cell proliferation perhaps by regulating progenitor cells in many tissues. Depending on the context, Hh can function as a morphogen, a survival factor, a mitogen, and even a guidance molecule [3, 4]. Inappropriate activation of the Hh signalling pathway has been associated to the development of different types of cancers including skin (basal cell carcinoma), prostate, lung, pancreas, breast and brain [5–10]. Due to the biological relevance of CSCs and EMT in cancer invasiveness, metastasis [11], drug resistance [12, 13] and tumor relapse, it is not surprising the therapeutically potential to develop Hh inhibitors to silence the Hh signaling pathway in cancer therapy.

Hh inhibitors have been obtained in the last few years and already used in many clinical trials with some very promising results. Some of these inhibitors are: GDC-0449, LDE-225, BMS-833923, IPI-926, PF-04449913, TAK-441, GANT-61 and Cur-61414 [14, 15]. Interestingly, a family of dietary chemopreventive agents known as nutraceuticals have been found to prevent, delay and even delay tumorogenesis [16–20] by inhibiting multiple signalling pathways including Hh signalling. Among some of these agents are: curcumin, soy isoflavones, vitamin D, resveratrol and epigallocatechin-3 gallate.

3.2 Hedgehog Signalling Pathway

Over the last decade, the Hh signalling pathway has been the object of intense study (Fig. 3.1). Briefly, in the so called "canonical" signalling pathway [1, 2, 21], it is known that in the absence of any Hh ligand (Sonic (Shh), Desert (Dhh) or Indian (Ihh)), the receptor Patched (Ptc1) prevents activation of the protein Smoothened (Smo), a seven-transmembrane protein, by inhibiting its translocation into the primary cilium. If Hh is present, then Smo accumulates in the cilium in an active conformation that induces the activation of the Gli family of transcription factors (Fig. 3.2). In the "non canonical" signalling pathway, it was found that not all Hh signalling goes through Gli activation. In fact it works through functions of Ptch1 that are unrelated to its inhibitory activity (Type I) (Fig. 3.3) on Smo or, through Smo functions beyond Gli regulation (Type II) [22, 23] (Fig. 3.4).

The Hh family is formed by three secreted proteins: Sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh)). These proteins undergo multiple biochemical processes to become activated [23]. The first event occurs when the protein is cleaved, then the C-terminal domain of the Hh protein catalyzes an intramolecular cholesteroyl transfer reaction, resulting in a Hh ligand with a C-terminal cholesterol moiety. This modification allows the association of Hh with cellular membranes, facilitating the final necessary modification when a palmitoyl moiety is added to the N-terminus of Hh by the transmembrane acyltransferase [23]. This results in an active Hh molecule that consists of a double lipid-modified signalling

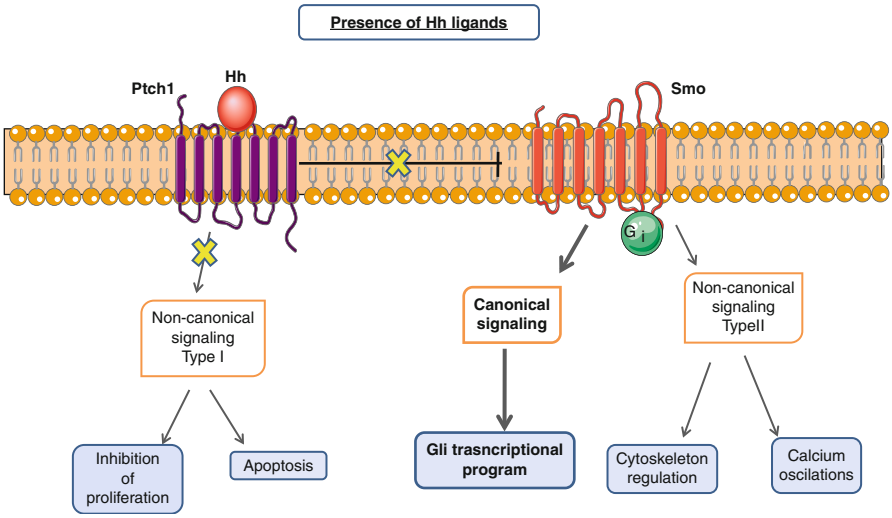

Fig. 3.1 The Hh signaling network. Hh binding to Ptch1 modulates one or more of the above modules: non-canonical type I Hh signaling through Ptch1; canonical Hh signaling through Ptch1, Smo and Glis; and non- canonical type II signaling through Smo and Gi proteins. *Yellow crosses* indicate inhibition of the pathway in the presence of Hh proteins. *Hh* hedgehog, *Ptch1* patched1, *Smo* smoothened, *Glis* glioma-associated oncogene family zinc finger transcription factor

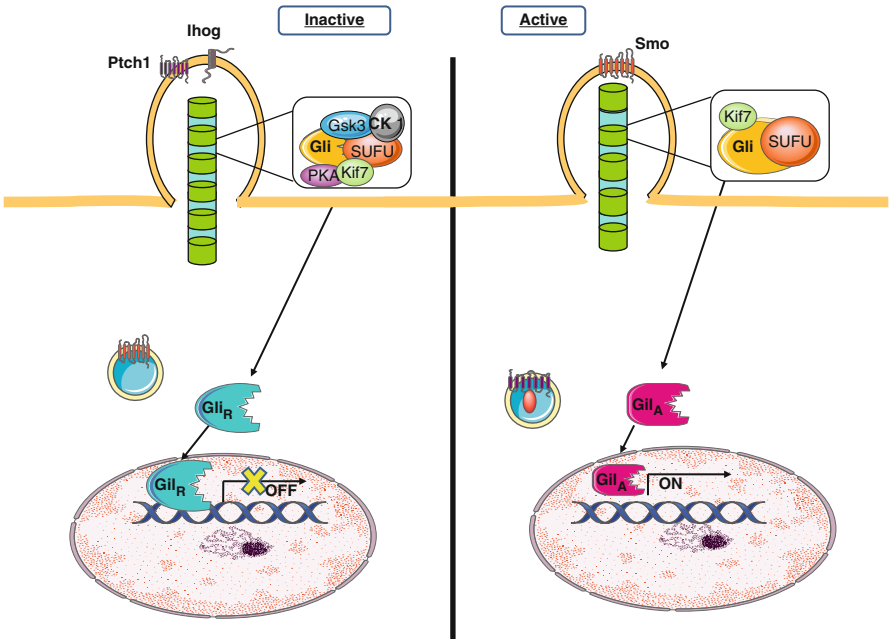

Fig. 3.2 The canonical Hh signaling pathway. In vertebrates Hh signaling take place on primary cilia. When Hh ligand binds to receptor complex (formed by Ptc and an Ihog coreceptor), Smo translocates to the plasma membrane and to the primary cilium, where it regulates Gli proteins

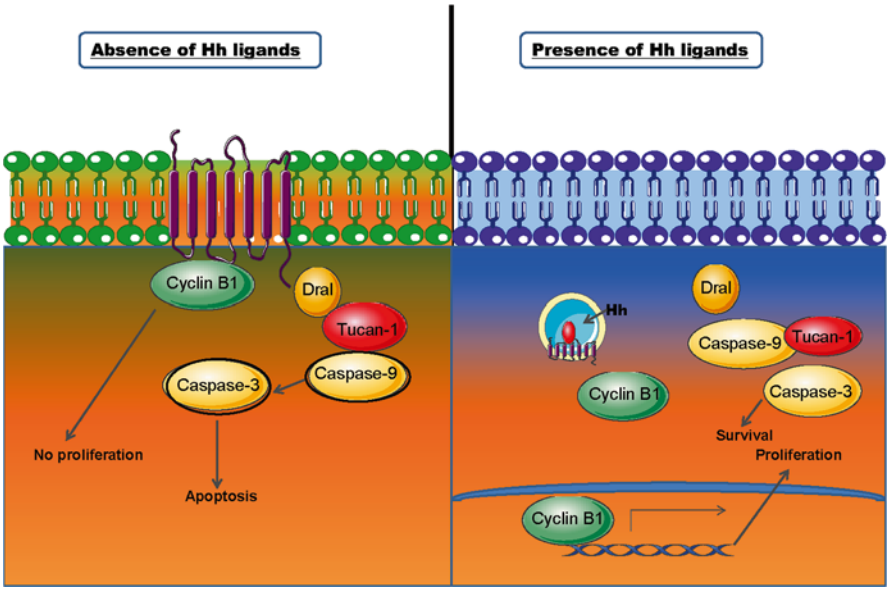

Fig. 3.3 Type I non-canonical Hh signaling. In the absence of a Hh ligand (*green*) Ptch1 interacts with cyclin B1 and a proapoptotic complex that includes caspase-9, the CARD (Caspase-associated recruitment domain) containing protein Tucan-1 and protein Dral. Hh binding (*blue*) disrupts the interaction of Ptch1 with cyclin B1 and the proapoptotic complex, likely through a conformational change in Ptch1, leading to increased survival and proliferation. *Black* highlighted caspase indicate activation

molecule. An important structure in many eukaryotic cells is the presence of cilia, which are tail-like projections of the cell membrane and have a biological relevant role in the components of the Hh signalling pathway [24]. Two important transmembrane proteins of the Hh signalling are Patched (Ptch) and Smoothened (Smo) and, both show Hh-dependent trafficking in the cilia [23, 24]. In the absence of Hh, the molecule Ptch inhibits (catalytically) the activity of the other transmembrane protein Smo acting as a receptor-like protein [25]. It is known that Smo is regulated by a small molecule that at the same time is also regulated by Ptch in terms of distribution and concentration. If Smo is not activated, then the glioma-associated oncogene family zinc finger (Gli) transcription factor in a complex with Fused and the suppressor of protein fused (SUFU, an important negative regulator of Hh signalling) is prevented from entering the nucleus. Moreover, without active Gli in the nucleus, the transcription of Hh target genes is suppressed. Three types of Gli transcription factors are present in mammalian cells: Gli1, Gli2 and Gli3. The three with different functions. Gli1 is an activator of Hh target genes, Gli2 Functions predominantly as an activator whereas Gli3 functions mainly as a repressor. Hedgehog signals are fine-tuned regulated based on positive feedback loop via Gli1 and negative feedback loop via Hhip1 (Hedgehog-interacting protein), PTCH1, and PTCH2. When Hh binds to Ptch it results in a loss of Ptch activity, and the consequent activation of Smo, which transduces the final Hh signal to the cytoplasm. The same activation of Smo causes a dissociation of the SUFU-Gli complex. Subsequently,

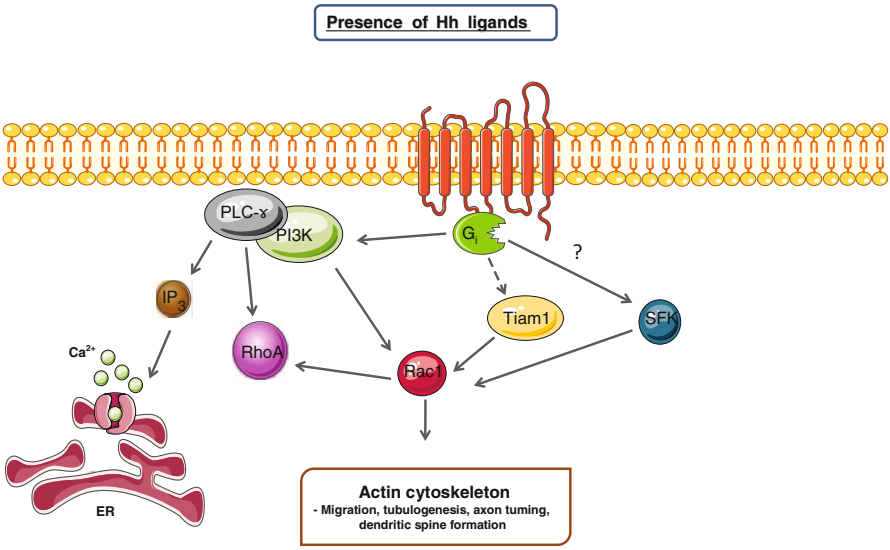

Fig. 3.4 Type II non-canonical Hh signaling. Smo regulates the actin cytoskeleton through RhoA and Rac1- small GTPases. This regulation occurs in a context-specific manner, through Gi, proteins and PI3K in fibroblast, and through Tiam1 or though the Src kinase family (*SFK*) members Src and Fyn in neurons. In addition, Smo stimulates calcium release from the endoplasmic reticulum (*ER*) in spinal neurons through Gi and PLC- Ð dependent channels

this leads to nuclear translocation and activation of Gli 1 and Gli2 transcription factors and degradation of Gli3 [23, 24]. Activated Gli promotes transcription of Hh target genes such as Ptch, Wnt, bone morphogenetic protein (BMP), Snail, Slug and Twist just to mention some of them. Furthermore, activated Hh ligands including Shh, Dhh and Ihh stimulate Gli transcription factors which form the final effectors of the Hh signalling pathway.

3.2.1 The Hedgehog Signaling Pathway Cross-Talk with Other Cell Regulator Pathways

For the cellular homeostasis is required that the multiple signalling pathways form a network to maintain normal signal transduction and control cellular functions [26]. There are many signalling pathways that cross- talk to the Hh signalling pathway such as Wnt/ β-catenin, Notch and TGF-β/BMP pathways. Interestingly, these same pathways are implicated in tissue morphogenesis, cellular homeostasis and stem-cell renewal [21, 26, 27]. In cancer, aberrations in the cross-talk between Hh and these regulator pathways could play an important role in the formation and maintenance of cancer stem cells (CSCs) [27] cancer invasion, metastasis and cancer relapse. Both, the Hh and the Wnt signaling pathways are evolutionary well preserved in humans implying not only a critical role for these proteins in cell and organ development but,

a role in carcinogenesis if these pathways are deregulated or aberrant. Many studies have shown similarities between the Hh and the Wnt signalling pathways [28]. For example, both pathways are activated by G-protein-couple receptors (Smoothened or Frizzled) [29, 30]. Signaling of Hh or Wnt, inhibits phosphorylation-dependent proteolysis of a key effector that converts a DNA-binding protein from a repressor to an activator factor of transcription. In addition, activation of Gli stimulates the transcription of Wbt ligands, suggesting that Wnt signalling may well be a downstream target of Hh signalling. Paradoxically, it has been found that molecules of Wnt signalling such as glycogen-synthase kinase (GSK)-3β could also regulate molecules of the Hh signalling pathway. It is also known that this molecule phosphorylates and stabilizes Sufu which leads to the inhibition of Hh activation [31]. It is then very plausible that any alteration in the crosstalk between Hh and Wnt signalling can be biologically relevant in cancer development.

The other important pathway that cross-talks to the Hh signalling pathway is the Notch pathway and its proteins. Similar to Hh and Wnt, the Notch family of proteins is evolutionary very well conserved in humans. They have similar properties as the other two, Hh and Wnt, in terms of organ development, cellular homeostasis and stem-cell renewal. It has been suggested that the crosstalk between Hh and Notch can increase the expression of hairy and enhancer of split 3 (Hes3) a molecule that participates in Notch signalling and Shh leading to the survival of stem cells [27]. Moreover, the signaling of Hh together with Wnt and Notch the signaling pathways could regulate self-renewal and differentiation of breast cancer stem cells or early progenitor cells [32]. Finally, Hh can also crosstalk with TGF- β and Wnt signalling to participate in the process of bone development and, in the acquisition of the EMT phenotype. The first property important for metastasis and the second for invasiveness.

3.2.2 Hedgehog in Cancer

It is well documented that alterations of Hedgehog signaling pathway have been associated to human cancers. Some somatic mutations which activate the Hh signaling pathway have been detected in many cancers [4–11]. Excessive positive feedback or collapsed negative feedback of Hh signaling due to genetic or epigenetic alterations leads to carcinogenesis. Many properties associated to carcinogenesis have been reported during Hh signaling. These are the most relevant: Hb signaling induces cellular proliferation through upregulation of Cyclin D/E, FOXM1 and, N-Myc. Hh protein signals directly upregulate WNT and JAG2. Hh signals indirectly upregulate mesenchymal BMP4 via FOXF1 or FOXL1. In addition, Hh signals induce stem cell markers such as CD44, CD133, BMI1 and, LGR5 based on cross-talk with WNT and possibly other signals. Furthermore, Hh protein signals upregulate BCL2 to promote cellular survival, SNAI1 (Snail), SNAI2 (Slug), ZEB1, ZEB2 (SIP1), TWIST2, and FOXC2 to promote epithelial-to-mesenchymal transition (EMT), and PTHLH (PTHrP) to promote osteolytic bone metastasis [1, 3, 4, 21].

Hh is aberrantly activated in glioma, medulloblastoma, basal cell carcinoma (BCC), esophageal cancer, lung cancer, breast cancer, pancreatic cancer, gastric cancer, and other tumors. In BCC, the most extensively studied cancer which occurs as a sporadic form or inherited form (basal cell nevus syndrome, BCNS). Using genetic linkage studies, mutations of Ptch have been identified in BCNS. In addition, approximately 90 % of sporadic BCC present also mutations also in Ptch [33–35]. The mutation induces lose of Ptch function and as a consequence Smo is no longer suppressed, leading to the continuous, persistent activation of Hh signalling during BCC development. Furthermore, gene silencing of Gli2 is able to inhibit BCC cell growth ***in vivo*** [36], strongly suggesting the importance of persistent Hh activation in BCC development.

With regards pancreatic cancer, Gli1 plays an important role in cell proliferation of pancreatic ductal adenocarcinoma. Gli1 promotes cell migration and invasion of pancreatic cancer cell through mucin 5, subtypes A and C mediated attenuation of E-cadherin [37]. It has been found that the expression level of Shh is higher in pancreatic adenocarcinoma than in non-malignant pancreatic tissues [38]. Furthermore, the expression pattern of Shh, Gli1 and Ptch has been shown to be associated with pathological and clinical features of pancreatic cancer. All these preclinical and clinical data strongly suggest an important role for Hh proteins in the malignant process of this tumor. Interestingly, while it is well known that pancreatic cancers have a dense fibrotic stroma, now it is known that fibroblasts from human primary pancreatic cancer over-expresses Smo, which could transduce Shh signal causing the activation of Gli1 promoting fibroblast proliferation [39]. It is for this reason that the use of inhibitors of Smo and Shh can be justified to facilitate drug delivery and optimize cancer treatments [40].

In prostate cancer, there is epithelial expression of Hh ligand during prostatic gland development [41]. It has been found that increased Hh signaling is associated with prostate cancer progression. Moreover, activation of Hh signaling has been reported to accelerate cancer growth [41, 42]. Nevertheless, there have been reports of prostatic cells lacking active canonical Hh signaling. Further studies are required to elucidate this important aspect of Hh signaling and prostate cancer.

In colon cancer, esophagus and in hepatic cancer, there has been found an association between activation of the Hh signaling pathway and cancer progression due to the formation and maintenance of CSCs and induction of EMT [43–45]. The same is true for lung cancer. In this case however, it has been found that chronic exposure of TGF-β to NSCLC cell lines (A540 and H2030) induces EMT [46], leading to upregulation of Shh both at the RNA and at the protein level, causing activation of Hh signaling pathway [47]. In addition, it has been reported that the aggressiveness of EMT-transformed cells was dramatically reduced by Shh knockdown or by using Hh inhibitors implying and confirming that activation of Hh signaling by TGF-can induced EMT and aggressiveness to the cancer cells [46]. In breast cancer, the same biological activities occur as with other cancers. In this case, activation of the Hh signaling pathway promotes the progression of non-invasive breast cancer to invasive breast cancer [48]. It also appears that Hh ligands secreted by breast cancer cells may induce differentiation and activation of osteoclast promoting breast cancer bone metastasis by upregulating osteopontin, and MMP-9 [49].

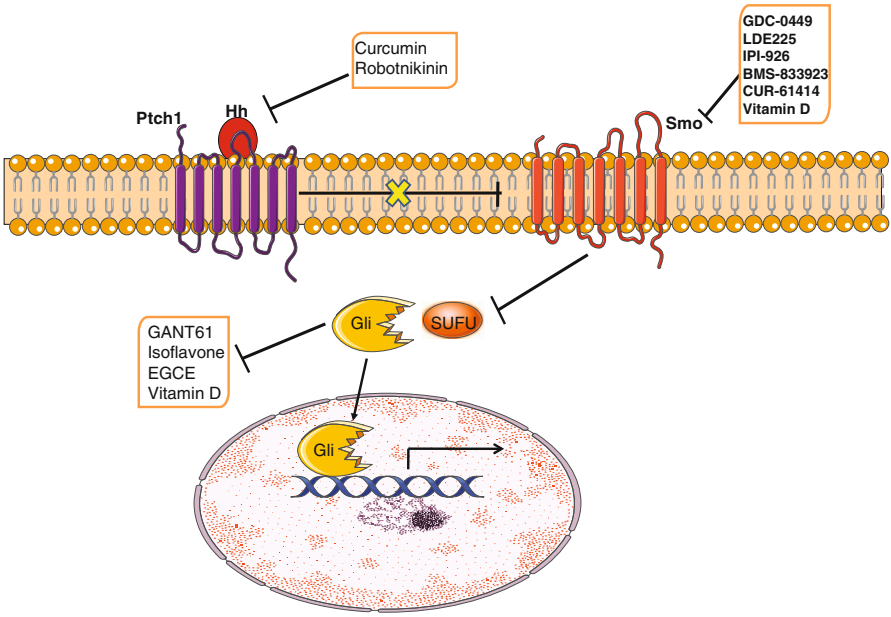

Fig. 3.5 Effects of small-molecule Hedgehog inhibitors and nutraceuticals on the Hedgehog signaling pathway

3.3 Cancer Treatment with Hedgehog Inhibitors

The important body of information obtained from preclinical studies, implying a major role for the Hh signaling pathway in cancer led to the design and synthesis of many small-molecules inhibitors of Hh for cancer treatment. In this review will discuss those in more advanced clinical development: GDC-0449, LDE225, IPI-926, BMS 833923, TAK-441, PF-04449913, Cur-61414 and GANT61 (Fig. 3.5).

3.3.1 GDC-0449 (Vismodegib®)

Vismodegib® (GDC-0449) is first-in-class, oral, selective Hedgehog pathway inhibitor. It is a small-molecule that binds and selectively inhibits Smo1–4. It has a molecular weight of 421.3 g/mol. It is given orally, once daily dosing (150 mg/day) [50]. The combination of ponatinib (a pan–ABL1 kinase inhibitor) with Hh Smo inhibitor GDC-0449 (Vismodegib®) was observed to eliminate therapy-resistant NOD/SCID re-populating T315I BCR-ABL1 positive leukemia cells in vitro and in vivo [51]. These results suggest an important role for GDC-0449 for controlling minimal residual disease in BCR-ABL1 positive leukemia [51]. A phase 1 clinical trial was conducted in patients with basal-cell carcinoma (BCC). In BCC several mutations

in Hh pathway genes, primarily genes encoding ***Ptch1*** and ***Smo*** have been identified. GDS-0449 was assessed in 33 patients with metastatic or locally advanced BCC for safety, pharmacokinetics and clinical responses [52]. The median duration of the study treatment was 9.8 months. Of the 33 patients, 18 had an objective response to the HhI GDC-0449, a 55 % response rate. Of the patients who had a response, 2 had a complete response and 16 had a partial response. The other 15 patients had either stable disease (11 patients) or progressive disease (4 patients) [52]. Grade 3 adverse events were reported in 6, including four with fatigue, two with hyponatremia, one with atrial fibrillation and one with muscle spasm. These excellent results are extremely provocative particularly since they were obtained in a phase 1, a very rare event in drug development. Another phase 1 clinical trial was conducted using the same compound in patients with pancreatic cancer (n = 8), medulloblastoma (n = 1) and with other type of cancers (n = 17). Tumor responses were observed in the patient with medulloblastoma and 14 patients presented stable disease as their best response [5, 53]. Because on these results several phase II trials are being conducted. In patients with medulloblastoma treated with GDC-0449, initially they present a rapid regression of the tumor and reduction of the symptoms [54] However, patients ultimately relapsed with a D473H resistance mutation in Smo which is the target of the new compound. Other studies have found that the resistance can occur downstream of Smo [55, 56]. Interestingly and clinically relevant is the fact that these medulloblastoma resistance patients retained their sensitivity to PI3K inhibition suggesting that for better clinical responses it may be necessary to combine a Hh with an Akt signalling inhibitors [56]. Several clinical trials are being conducted using GDC-0449 in many other type of cancers, ovary and colorectal among them.

3.3.2 LDE225

LDE225 is another small-molecule designed to inhibit specifically Smo in the Hh signalling pathway. A phase I clinical trial evaluating the safety, tolerability and effect of this compound in cream preparation to apply topically to nevoid basal cell carcinoma syndrome (NBCCS) with BCC has been performed in 13 patients. The results showed that LDE225 induces complete clinical responses in 3 patients, a partial response in 9 cases, and no response in one case. The treatment was well tolerated and it did not induce skin irritation [57]. Mean volume reduction of 40.8 % was seen in the experimental arm (LDE225) compared with 9.1 % in the vehicle/control arm. Clinical response was found to be correlated with a reduction of the RNA levels of Gli1, Gli2 and Ptch. Another phase I clinical trial in 25 patients with advanced solid tumors were treated with LDE225 orally once daily on a continuous 28-day dosing schedule. Interestingly, a dose-dependent reduction of Gli1 RNA was identified. One patient with medulloblastoma presented a partial response that was maintained for 4 months and 5 patients with different types of cancer were treated for more than 4 months with good tolerance to the compound [58]. As it was the case for GDC-0449, patients undergoing treatment with LDE225 developed resistance. The mechanisms

of such resistance appear to relate to an amplification of Gli2 and point mutations to Smo which can result in a reactivation of the Hh signalling pathway.

3.3.3 IPI-926

The compound IPI-926 is also a molecule produced to inhibit Smo of the Hh signalling pathway. Interestingly, this molecule represents an active analogue of the naturally occurring alkaloid Veratrum cyclopamine. In a phase 1 clinical trial IPI-926 in patients with advanced/metastatic solid tumors (including BCC) it was seen a 32 % partial response in BCC patients (4/17) with good tolerability. In a pancreatic mice model of pancreatic ductal adenocarcinoma refractory to gemcitabine, IPI-926 was given since this compound can deplete tumor stroma by inhibition of Hh signalling. It is well known that pancreatic cancers have a dense fibrotic stroma making very difficult the delivery of any drug to the tumor. IPI-926 was given with gemcitabine resulting on a higher intratumoral vascular density and intratumoral concentrations of gemcitabine. That was associated with a transient stabilization of the disease [40]. Currently, there are several ongoing trials phase I/II using this new compound in pancreatic cancer together with gemcitabine and in unresectable, advanced chondrosarcoma.

3.3.4 BMS-833923

This compound is a small molecule orally bioavailable Hh inhibitor. The Hh signalling inhibition is achieved by binding to Smo. Several phase 1 clinical trials are been conducted on multiple myeloma, and chronic myelogenous leukemia. In addition, a phase Ib clinical trial is being conducted in patients with unresectable metastatic gastric cancer, gastroesophageal and esophageal adenocarcinoma using BMS-833923 in combination with cisplatinum and capecitabine as first line therapy.

3.3.5 GANT61

This new compound is entering clinical trials. It is a Hh inhibitor that targets Gli1 and Gli2. The effects of GANT61 on human colon cancer cell lines, GC3/cl and HT-20, have been analyzed. The compound causes G1/S arrest with upregulation of p21 and p15. In addition, GANT61 treatment eliminates the clonogenicity of human colon carcinoma cell lines and decreased Bcl-2 expression, most likely via inhibition of Gli1 and Gli2 [59]. In human cell lines of bladder transitional cell carcinoma GANT61 was able to decrease cell invasiveness by inhibiting Gli2 [60]. In oral squamous cell carcinoma GANT61 causes downregulation of Gli1 expression, inhibited cell proliferation, cell migration and also induced G1 arrest and apoptosis [61].

3.3.6 Other Hedgehog Signalling Inhibitors

There have been many Hh signalling pathway inhibitors designed and already tested in preclinical studies and in phase I clinical trials. Of interest at the present moment there are three more: TAK-441, PF-04449913 and Cur-61414. The first one, TAK-441 is an oral Hh inhibitor that is currently being tested in a Phase 1 clinical trial including patients with advanced nonhematological tumors, in particular in patients with metastatic and advanced solid tumors including BCC. The second one is PF-0449913, another orally small molecule bioavailable Sonic-Hh signaling pathway inhibitor. Currently is being tested in a phase I clinical trial in haematological malignancies. This compound will be tested in patients with chronic myeloid leukemia in combination with Dasatinib or Bosutinib. The third one, Cur-61414, is also a small molecule inhibitor of the Hh signalling pathway. It has been shown that this compound can abolish the Hh signalling activation produced by the oncogenic mutations in Ptch. It has been observed that it inhibits Smo, leading to the downregulation and consequent inactivation of Hh signalling. It appears that this compound can suppress proliferation and induce apoptosis of nests of basaloid cells in basal cell carcinoma models. No such an effect was observed on normal skin cells [62]. If this effect can be confirmed, Cur-61414 may well represent a new agent for the treatment of BCC.

3.4 Nutraceuticals: A New Class of Regulators of Hedgehog Signaling

A new class of regulators of the Hh signaling pathway has been recently identified within some dietary chemopreventive agents known as nutraceuticals. These compounds could inhibit the growth of cancer cells and induce apoptosis through the regulation of many signaling pathways including the Hh signalling [63] (Fig. 3.5). Due to their relatively low toxicity, they are considered in future therapeutic strategies combined them with known cancer treatment agents. In this review, only those with wider experience will be discussed: curcumin, soy isoflavones, epigallocatechin-3gallate (EGCG), resveratrol and vitamin D.

3.4.1 Curcumin

Curcumin is the principal active component of Indian curry spice turmeric extracted from ***Curcuma longa***. It is well known for its anti-oxidant, anti-inflammatory, anti-atherogenic and anti-carcinogenic properties [64, 65]. Curcumin has been observed to have anti-cancer activities both ***in vitro*** and ***in vivo***. Recently, a regulatory effect on Hh signalling has been observed in several types of cancer. It has been shown that curcumin has a suppressive effect on medulloblastoma cell proliferation, inducing

cell cycle arrest at G2/M phase through the downregulation of Shh and Gli1 [66]. In addition, curcumin reduces the protein level of b-catenin and its downstream targets, such as c-Myc and Cyclin D strongly suggesting that curcumin may well block the cross-talk between Hh and Wnt signalling pathways [63]. Moreover, curcumin can downregulate Bcl-2 what makes this compound a good candidate to use it in combination with chemotherapeutic anti-cancer agents in particular in medulloblastoma. The poor bioavailability of this compound ***in vivo*** may hamper its truly potential as an anti-cancer agent. In prostate cancer, it has been observed that curcumin inhibits Gli1 mRNA expression and downregulates Gli reporter activity which was associated with an important inhibition of prostate cancer cell growth. Recently, it has been observed in brain tumors that curcumin could inhibit the expression of Gli1 resulting in the downregulation of cell growth and stem cell phenotype. Curcumin induces cell death and restores tamoxifen sensitivity in the antiestrogen-resistant breast cancer cell lines MCF-7/LCC2 and MCF-7/LCC9 [67]. Recently, synthetic analogs of curcumin and nanocurcumin have been elaborated which are the next generation targeted therapy with curcumin in cancer [68].

3.4.2 Soy Isoflavones

Isoflavones are mainly found in the ***Leguminosae*** family. Many foods are rich in these compounds: soy, lentils, beans and chickpeas. Soybeans contain rich amounts of isoflavones. Three main isoflavones including genistein, glycitein and daidzein are obtained from soybeans and, in most soy protein products. Studies on isoflavones have shown a protective effect against various types of cancer. Genistein has been by far the most studied compound due to its known anti-cancer properties. These effects have been observed in prostate cancer, gensitein can inhibit Gli1 mRNA expression and Gli1 reporter activity, both effects associated to an inhibitory effect on prostate cancer growth. In addition, genistein inhibits the stemness properties of prostate cancer cells through targeting Hedgehog-Gli1 pathway [69, 70].

3.4.3 Epigallocatechin-3 Gallate (EGCG)

Epigallocatechin-3gallate (EGCG) is a catechin found in tea green known for its anti-cancer properties [71]. Consumption of green tea has been associated with a protection against cancer. It is the most potent constituent found in green tea for the inhibition of carcinogenesis and oxidative stress among the catechin family of compounds [71]. EGCG induces apoptosis and suppresses proliferation by inhibiting the human Indian Hedgehog pathway in human chondrosarcoma cells [72]. It has been observed also that EGCG decreases Gli1 mRNA expression and inhibits Gli1 reporter activity resulting in the inhibition of prostate cancer cell proliferation.

In a recent study, EGCG inhibited self-renewal capacity of pancreatic CSCs and synergized with quercetin, a major polyphenol and flavonoid commonly detected in many fruits and vegetables. EGCG inhibited also the expression of pluripotency maintaining transcription factors (Nanog, c-Myc and Oct-4) and self-renewal capacity of pancreatic CSCs. Inhibition of Nanog by shRNA enhanced the blocking effects of EGCG on self-renewal capacity of CSCs. EGCG inhibited cell proliferation and induced apoptosis by inhibiting the expression of Bcl-2 and XIAP and activating caspase-3. Furthermore, EGCG also inhibited the components of SHh pathway (smoothened, patched, Gli1 and Gli2) and Gli transcriptional activity. In addition, EGCG inhibited EMT by abolishing the expression of Snail, Slug and ZEB1, and TCF/LEF transcriptional activity. These effects correlated with significantly reduced CSC's migration and invasion, suggesting the blockade of signaling involved in early metastasis. Furthermore, combination of quercetin with EGCG had synergistic inhibitory effects on self-renewal capacity of CSCs through attenuation of TCF/LEF and Gli activities [73].

3.4.4 Resveratrol

Resveratrol is a major constituent of traditional Asian medicinal herbs and red wine and besides being a potential hypolipidemic drug it has anti-inflammatory and antioxidative properties. With regards cancer, it has been observed to inhibit proliferation and to induce apoptosis through the hedgehog signaling pathway in pancreatic cancer cells [74]. In addition, resveratrol inhibited growth of 4T1 breast cancer cells in a dose- and time-dependent manner [75]. ***In vivo***, however, resveratrol had no effect on time to tumor take, tumor growth, or metastasis when administered intraperitoneally and has no growth-inhibitory effect on 4T1 breast cancer ***in vivo***. However, resveratrol has shown to be able to suppress the development and progression of prostate cancer in TRAMP mice [76].

3.4.5 Vitamin D

From epidemiological and experimental studies it is known that a higher intake of vitamin D either from food or in supplements, and higher levels of vitamin D in blood has been associated with a protective effect on cancer risk and cancer-associated mortality [77]. Potential mechanisms of action include inhibition of the Hh signaling pathway and upregulation of nucleotide excision repair enzymes. However, this association – skin cancer and vitamin D- is complicated by ultraviolet B radiation. The same spectrum of ultraviolet B radiation that catalyzes the production of vitamin D in the skin also causes DNA damage that can lead to epidermal malignancies [77]. Since, vitamin D inhibits the activation of Hh signaling, vitamin D may serve as a potential treatment option for those human cancer that present activated Hh signalling [77, 78].

Recent studies indicate a potential role of vitamin D and its receptor (VDR) in protecting against the development of epidermal tumors [79, 80]. One such study found mice lacking the VDR were quite sensitive to epidermal tumor formation following the administration of the carcinogen DMBA. A more recent study showed that these mice were similarly more sensitive to tumor formation following UVR [79, 81]. It was observed that observed the transcriptional activity of beta-catenin was increased in keratinocytes lacking the VDR. These results lead to the hypothesis that the VDR with its ligand 1,25(OH)(2)D(3) functions as a tumor suppressor with respect to epidermal tumor formation in response to UVR by regulating Hh and beta-catenin signalling [81]. In BCC cells, it was observed that vitamin D3 inhibited cell proliferation through the downregulation of Gli1 expression. Furthermore, topical vitamin 3 treatment of existing murine BCC tumors significantly suppressed the expression of Ki67 and Gli1 implying that topical vitamin D3 could be an effective anti-BCC compound targeting Hh signalling pathway [80, 81].

Vitamin D and its analogs have shown antitumor activity in human renal cell carcinoma [82], vitamin D3 was found to inhibit pancreatic adenocarcinoma cell growth specifically through inactivation of Smo and the downstream Hh pathway, rather than activation of the vitamin D3 receptor. However, it was not found to be effective against tumor cell growth in **in vivo** models [84].

Conclusion

The Hh signalling pathway plays relevant biological functions in cell differentiation and organ formation during embryonic development. In addition, it participates in the formation and maintenance of cancer stem cell (CSC) and in the acquisition of epithelial-mesenchymal transition (EMT) (Table 3.1). Due to its role in CSCs and EMT phenotype, Hh signalling factors are involved in cancer invasion, metastasis, the development of drug resistance and cancer recurrence. For the last few years, Hh signaling pathway has been the focus of drug development targeting Hh factors for cancer prevention and treatment. Several small molecules have been designed to inhibit Hh signalling pathway proteins: GDC-0449, LDE225, IPI-926, BMS 833923, TAK-441, PF-04449913, Cur-61414 and GANT61. Most of them being tested, currently, in clinical trials. Very promising results have been obtained in BCC using GDC-0449 (Vismodegib®). Hh signalling inhibitors have shown anticancer activity in preclinical as well as in clinical studies in a variety of tumors: prostate, medulloblastoma, pancreas, breast, colon, chondrosarcoma among others. Interestingly, several dietary chemopreventive agents of the so-called nutraceutical family (curcumin, soy isoflavones, vitamin D, resveratrol and epigallocatechin-3 gallate) have been shown to inhibit tumor growth through downregulation of the Hh signalling pathway. The future design of novel therapeutic strategies combining inhibitors of the Hh pathway with nutraceuticals to inhibit and regulate activated Hh could bring new and promising tools for cancer treatment.

Table 3.1 Relevant features of the Hedgehog (Hh) signalling pathway

1. The Hh pathway is important in cell differentiation and organ formation during embryonic development
2. Hh signalling normally remains inactive in most adult tissues
3. Reactivation of Hh signalling can results in tumourigenesis
4. The Hh signalling pathway plays important roles in stem cell maintenance, stemness, tissue repair and regeneration
5. There is cross-talk among Hh, Wnt, Notch and TGF-β/BMP pathways. These factors play important roles in tissue morphogenesis and stem cell renewal
6. Alterations in the Hh pathway have been found in human cancers and its activation has been associated with EMT and cancer progression
7. Small-molecules inhibitors have been designed to target Hh signalling pathway. Many of these agents have shown anti-tumoral effects. One of them GDC-0449 (Vismodegib®) has shown very promising results in BCC in clinical trials
8. A family of dietary chemopreventive agents (nutraceuticals) has been found to inhibit Hh pathway and to possess anti-tumor activities. Combination between Hh inhibitors and nutraceutical agents may represent a future new anti-cancer strategy

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Chapter 4
Wnt Pathway at a Glance: From the Deep of the Crypts to the Current Ways of Targeting

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Abstract Wnt/β-catenin signalling pathway is crucial for the formation of many tissues and organs during development. In recent years, this pathway has also been found to regulate the biology of stem cells in the intestine and probably in other organs in adult life. Abnormal activation of Wnt/β-catenin signalling, which controls the expression of a high number of genes, is critical for the initiation and progression of most colorectal cancers. In line with this, the gene expression signature induced by activation of the Wnt/β-catenin pathway defines the intestinal stem cells present at the bottom of the crypts and also colon cancer stem cells. This supports the importance of inhibitors of the Wnt/β-catenin pathway as potential agents in colorectal cancer therapy. However, the complexity, wide activity in the organism modulating the biology of several cell types, and characteristics of this pathway have delayed the identification of suitable targets and so, the development of such inhibitors that are only now reaching the clinic.

Keywords Wnt • β-catenin • Intestine stem cells • Colon cancer • Cancer stem cells • Wnt inhibitors

4.1 Wnt Factors

4.1.1 Introduction

Most mammalian genomes, including the human genome, harbor 19 Wnt genes, falling into 12 conserved Wnt subfamilies [1]. Wnt genes are present in multicellular animals throughout the animal kingdom, but not in single-cell organisms,

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suggesting that Wnt signalling may have played an important role in the evolutionary origin of multicellular organisms [2]. Members of the Wnt family regulate key developmental processes such as tissue patterning, cell proliferation, and cell migration among others [3]. In the adult, Wnt signalling is crucial for tissue homeostasis by regulating stem cell maintenance [4]. As a result of its global importance, deregulation of Wnt signalling is associated with several human pathologies, most notably cancer [1].

4.1.2 Wnt Factors Synthesis and Secretion

Wnt proteins are around 40 kDa in size (350–450 amino acids) and contain 23–25 conserved cysteines [5]. Wnts are highly hydrophobic proteins in part due to two lipid modifications; a saturated palmitate chain, attached to a conserved cysteine residue (Cys^{77} in mouse Wnt3a, the first Wnt to be purified) [6] and a monounsaturated palmitoleate chain on a conserved serine residue (Ser^{209} in mouse Wnt3a) [7]. Lipid modification is required for both the secretion and the signalling activity of Wnt proteins and may explain their low range distribution and predominantly autocrine-paracrine activity. The current consensus is that lipidation is important for the exit of Wnts from the endoplasmic reticulum (ER) of Wnt producing cells [8]. A good candidate for mediating this lipid modification is Porcupine (Porc), a multipass transmembrane protein in the ER that belongs to the membrane-bound O-acyltransferase family. Depletion of Porc leads to a complete block in Wnt secretion and accumulation of Wnts in the ER [9]. Mutations and deletions in the human gene (*PORCN*) are associated with several developmental disorders, such as focal dermal hypoplasia and osteopathia striata [10, 11].

The seven-transmembrane protein Wntless (Wls, also known as Evenness interrupted/Evi or Sprinter) is a critical component of the Wnt secretion machinery. Wls binds Wnts and localizes to the Golgi, the plasma membrane and endosomes [12, 13], indicating that it functions downstream of Porc in the secretory pathway. In the absence of Wls, Wnts accumulate in the Golgi [14], suggesting that Wls functions as a sorting receptor that transports Wnts from the Golgi to the cell surface for release. Multiple evidences suggest that after Wnt secretion Wls is endocytosed and recycled to take part in multiple rounds of secretion [8]. An intracellular trafficking complex called the retromer is involved in Wls transport from endosomes to the Golgi as part of this recycling pathway [14, 15]. In retromer mutants, Wls fails to be transported to the Golgi and is instead degraded in the lysosomal system, possibly explaining the strong Wnt signalling defect observed in retromer mutants. Therefore, the retromer may be at a key control point in the Wnt secretion pathway, deciding whether Wls is recycled or degraded and thereby setting the level of Wls available for Wnt secretion [8].

Lipidation is also essential for Wnt activity. Recently, Chris Garcia's lab obtained the first crystal structure of a Wnt protein (*Xenopus laevis* Wnt8, XWnt8) bound to the cysteine-rich domain (CRD) of the mouse Wnt receptor Frizzled8. XWnt8

presents two domains with fingerlike protrusions, resembling a thumb and index finger that pinch the CRD, which contains a hydrophobic groove that interacts with the palmitoleate moiety on the Wnt molecule [16].

Wnt ligands are also highly glycosylated proteins [8], and sequence comparison suggests that all Wnt family members carry at least one N-linked glycan. The precise role of the glycosylation, however, has not yet been determined, and despite promoting both secretion and signalling it does not appear to be strictly necessary for either function [17].

4.2 Wnt Signalling Pathways

4.2.1 General

Secreted Wnt proteins can activate different signalling pathways. The Wnt/β-catenin or canonical pathway is the most studied, and is highly conserved throughout evolution. All other pathways triggered by Wnt proteins are much less known and have been globally named non-canonical Wnt signalling pathways.

4.2.2 Wnt/β-Catenin or Canonical Pathway

The Wnt/β-catenin pathway controls the intracellular levels of the β-catenin protein. β-catenin is involved in cell-cell adhesion but it also behaves as a co-activator for a family of transcription factors, named LEF/TCF, that control the expression of a large set of genes whose products are involved in a plethora of cellular processes. In the absence of Wnt proteins, β-catenin is located at the epithelial cells *adherens junctions* bound to the cytoplasmic tail of E-cadherin. β-Catenin levels in the cytosol are kept low due to the activity of a multiprotein complex known as "the β-catenin destruction complex". Components of this complex include the scaffold proteins encoded by the tumor suppressor genes *APC* (*adenomatous polyposis coli*) and *Axin*, and the serine/threonine kinases casein kinase 1α (CK1α) and glycogen synthase kinase 3 (GSK3). Free cytosolic β-catenin, either newly synthesized protein or released from *adherens junctions*, is recognized by Axin and APC, both of which can directly interact with β-catenin. Then, CK1α phosphorylates β-catenin at Ser^{45}, priming the sequential phosphorylation of Thr^{41}, Ser^{37}, and Ser^{33} by GSK3 (preferentially by GSK3β) [18]. Next, phosphorylated β-catenin interacts with the ubiquitin machinery, although it is not yet clear whether β-catenin has to leave the destruction complex for this interaction [1]. Ser^{33} and Ser^{37} phosphorylated β-catenin is recognized by β-TrCP and ubiquitinated by the Skp1/Cul1/F-box/β-TrCP (SCFβ-TrCP) E3 ubiquitin ligase complex. Ubiquitin-conjugated β-catenin is subsequently degraded by the 26S proteasome [19]. Alternatively, phospho-β-catenin can be ubiquitinated by the single unit E3 ligase Jade1 [20].

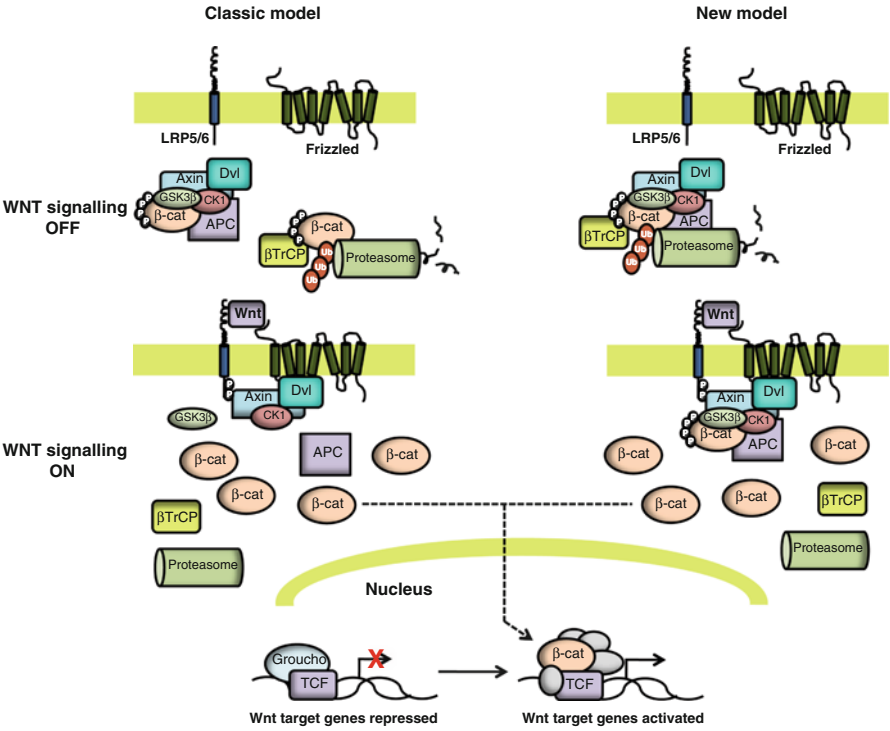

Fig. 4.1 The Wnt/β-catenin signalling pathway. In the classic model, in the absence of Wnt, the destruction complex binds and phosphorylates β-catenin that then leaves the complex to be ubiquitinated and degraded by the proteasome. Wnt induces the association of Axin with phosphorylated LRP5/6, which results in destruction complex disassembly and stabilization of β-catenin. In the new model, in the absence of Wnt, the destruction complex binds, phosphorylates, and ubiquitinates β-catenin and the proteasome recycles the complex by degrading β-catenin. Wnt induces the association of the intact complex with phosphorylated LRP5/6, but although the complex can still capture and phosphorylate β-catenin, ubiquitination is blocked. This results in saturation of the complex and accumulation of newly synthesized β-catenin. In both models, accumulated β-catenin enters the nucleus and binds LEF/TCF transcription factors, replacing transcriptional co-repressors such as Groucho. Then, β-catenin recruits transcriptional co-activators and histone modifiers to drive gene expression (Based on Clevers and Nusse [1])

Wnt proteins bind to a heterodimeric receptor complex, consisting of a Frizzled (Fz) and an LRP5/6 protein. The 10 mammalian Fz proteins are seven-transmembrane receptors and have large extracellular cysteine-rich domains [21] that provide a platform for Wnt binding [16, 22]. Fzs cooperate with a single-pass transmembrane molecule of the LRP family, either LRP5 or LRP6 [23]. Upon Wnt binding, LRP cytoplasmic tail is phosphorylated by at least two different kinases, GSK3 and CK1γ, generating *bona fide* docking sites for Axin. Interaction between phosphorylated LRP and Axin is facilitated by Dishevelled (Dvl), a scaffold protein recruited to the cytoplasmic domain of Fz upon Wnt binding [24]. Two different models try to explain why Axin binding to LRP results in β-catenin accumulation in the cytosol (Fig. 4.1). In the classical model, Axin binding leads to the disassembly of

the β-catenin destruction complex. As a result, β-catenin fails to be phosphorylated, and subsequently ubiquitinated and degraded; consequently, non-phosphorylated β-catenin is stabilized and accumulates in the cytosol [25]. In contrast, a recent model proposes that accumulation of β-catenin is the result of Wnt-mediated inhibition of ubiquitination of destruction complex-bound β-catenin. This would saturate destruction complexes with phosphorylated β-catenin molecules, allowing newly synthesized β-catenin to accumulate [26]. Nevertheless, independently of which model explains the mechanism, Wnt binding leads to an increase in the cytosolic levels of unphosphorylated β-catenin, a proportion of which enters the nucleus and engages DNA-bound LEF/TCF transcription factors. LEF/TCFs bind to specific DNA sequences referred to as Wnt-responsive elements (WRE: CCTTTGA/TA/T). In the absence of Wnt proteins, LEF/TCFs interact with transcriptional repressors such as Groucho/TLE1 [27, 28] preventing gene transcription. Association with β-catenin transiently converts LEF/TCFs into transcriptional activators of their target genes. The β-catenin C-terminus acts as a transcriptional activation domain [29] by binding histone modifiers such as CBP and Brg-1 [30].

The ultimate outcome of Wnt canonical signalling is determined by those genes whose transcription is controlled through β-catenin/TCF complexes. Numerous target genes have been identified in diverse biological systems and they are mostly tissue or developmental stage specific [31]. Only the *Axin2* gene is regarded as a global transcriptional target and therefore a general indicator of Wnt pathway activity [32]. For a comprehensive, updated overview of β-catenin/TCF target genes, we recommend Roel Nusse's Wnt homepage (http://www.stanford.edu/~rnusse/wntwindow.html).

Wnt/β-catenin signalling is regulated by numerous molecules that act at different cell locations. Extracellular inhibitors are secreted proteins that inhibit Wnt signalling at receptor level [33]. Secreted Frizzled receptor-related proteins (SFRPs) and Wnt inhibitory factor-1 (WIF-1) bind Wnt factors in solution, thus preventing their interaction with their plasma membrane receptors. The DICKKOPF (DKK) and Wise/SOST families bind LRP5/6 blocking the Wnt-Fz-LRP interaction. DKKs can also form a ternary complex with LRP5/6 and another class of high affinity DKK receptors named Kremen (Krm1/2), which induces rapid endocytosis and removal of LRP5/6 from the plasma membrane, thereby blocking Wnt/β-catenin signalling [34]. Recently, a new type of negative regulators of Wnt signalling has been discovered embedded in the plasma membrane. Tiki is a transmembrane protein that binds Wnt proteins and mediates their cleavage at a non-conserved site, resulting in loss of the N-terminal part of the protein. Cleaved Wnts no longer interact with their receptors but instead form disulfide-linked oligomers that are water-soluble [35]. ZNRF3 and RNF43 are two closely related transmembrane RING (TM-RING) finger proteins with E3 ubiquitin ligase function that regulate the stability and levels of cell-surface Fz and LRP5/6 proteins via ubiquitination and then internalization and lysosomal degradation of the receptor components leading to reduced Wnt signalling [36, 37]. In addition, an increasing number of intracellular inhibitors of Wnt signalling are known. Some of them function in the cytosol such as Naked or Axin2/Conductin [38–40], while others such as Chibby or ICAT block β-catenin action

within the cell nucleus either by direct binding or by binding to β-catenin partners and the promotion of β-catenin nuclear export [41, 42].

There are also several Wnt agonists, of which R-spondins are particularly relevant. The R-spondin family comprises four small secreted proteins defined by two N-terminal furin domains and a thrombospondin domain [43]. R-spondin proteins enhance Wnt signalling but only in the presence of Wnt ligands [44]. R-spondins bind to members of the Lgr family of seven transmembrane receptors (Lgr4/5/6). Recent evidence suggests that R-spondins contribute to stabilize Wnt receptors on the cell surface through the interaction of a Lgr family member and a ZNFR3/RNF43 member (see above) with R-spondins [36]. R-spondin binding to this Lgr/TM-RING receptor complex leads to the clearance of the TM-RING protein from the membrane (presumably via lysosomal degradation of the complex), with resultant increased levels of the Fz-LRP5/6 receptor complex and enhanced Wnt signalling [36, 45]. Interestingly, *Lgr5* is a Wnt target gene and it marks adult stem cells in a number of actively self-renewing organs, including the intestinal tract and the hair follicle [46–48] reinforcing the intimate connection between Wnt signalling and homeostasis of adult stem cells.

Deregulation or abnormal activation in adult life of the Wnt/β-catenin signalling pathway contributes to the emergence and progression of several types of human cancer. Cancer cells with mutationally activated Wnt pathway overexpress at least 20 target genes that activate proliferation including proto-oncogenes c-*MYC*, c-*JUN*, and *CCND1*/cyclin D1 [31].

4.2.3 Non-canonical Wnt Pathways

Under "non-canonical Wnt pathways" Wnt researchers have boxed up together different Wnt-triggered signalling pathways that are diverse in nature and are still evolving into an increasing number of, sometimes overlapping, branches [49].

The planar cell polarity (PCP) signalling pathway refers to the polarization of cells in an epithelial sheet and occurs, for example, during hair orientation or gastrulation [50]. Planar polarity signals are transmitted locally from cell to cell and mediated by Wnt signalling through Fz and Dvl independently of β-catenin. Dvl associates with multiple partners [51], among them the small GTPases Rho and Rac that upon activation of their effectors ROCK and JNK kinases, respectively, remodel the cytoskeleton [52], regulate dendrite growth [53] and control cell polarity and movement during gastrulation [54].

In the Wnt/calcium signalling pathway, Wnt/Fz signalling via heterotrimeric G proteins activates phospholipase C, leading to the generation of diacylglycerol and IP_3 which in turn generates calcium fluxes [55, 56]. Release of intracellular calcium activates several calcium-sensitive enzymes such as protein kinase C (PKC), calcium-calmodulin-dependent kinase II (CamKII), and the calcium-sensitive phosphatase calcineurin [49] which subsequently activate NFAT, NFκB, and cAMP response element-binding protein (CREB) transcription factors that

translocate to the nucleus and transcribe downstream regulatory genes [56]. The involvement of the Wnt/calcium pathway in dorsoventral patterning of early *Xenopus laevis* and zebrafish embryos is long known [57–59] but more recent evidences suggest that this pathway also plays roles in cancer, inflammation, and the nervous system [56].

In recent years, new alternative Wnt receptors have been discovered. Ryk (Related to tyrosine kinase) is a tyrosine kinase-like receptor that mediates Wnt-induced repulsion of axons, cell migration, neurite outgrowth and TCF activation [60–62], while Ror2 (also a receptor tyrosine kinase) seems to be the preferred receptor for Wnt5a [63], the prototype of a non-canonical Wnt. In mice, Ror2 and Wnt5a are spatially and temporally coexpressed during development [64] and mouse knockouts of Ror2 and Wnt5a exhibit partially overlapping phenotypes [64–66]. However, the biochemical evidence implicating Ror2 as a direct Wnt5a receptor remains inconclusive.

There are still many unclear issues regarding non-canonical Wnt signalling and its involvement in human disease. Interestingly, it has been recently proposed that non-canonical Wnt signalling might have a dual function in cancer progression, inhibiting early stages by antagonizing the Wnt/β-catenin pathway but enhancing later stages such as migration and invasion of cancer cells to promote metastasis [67].

4.3 Wnt/β-Catenin Pathway and Disease

4.3.1 Introduction

Upon the initial discovery of mutations in *APC* gene in familial adenomatous polyposis, a hereditary cancer syndrome, mutations in many components of the Wnt/β-catenin pathway have been found in a large number of human diseases [1]. The significance of these alterations is best characterized in the intestinal tract.

4.3.2 Wnt/β-Catenin Pathway in Normal Intestine

The intestinal tract is anatomically divided into the small intestine (duodenum, jejunum and ileum) and the large intestine or colon. The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals: the small intestine of the mouse completely renews every 3–5 days. The absorptive epithelium of the small intestine is ordered into villi and crypts of Lieberkühn. Each crypt contains around 250 cells and generates a similar number of new cells each day [68]. Homeostasis of the intestinal epithelium is maintained by an intestinal stem cell (ISC) compartment that resides at the bottom of the crypt. These ISCs give rise to proliferating progenitor or transit amplifying (TA) cells that rapidly divide four to five times

before undergoing differentiation upon crossing the crypt-villus junction [69]. The differentiated cell types of the intestinal epithelium are well defined in terms of marker expression and morphology. Absorptive enterocytes, mucus-secreting goblet cells and enteroendocrine cells occupy the villi while Paneth cells, which secrete antimicrobial products and provide stem cell niche signals, reside only at crypt bottoms of the small intestine [70]. The mucosa of the colon has a flat surface epithelium instead of villi. Deep secretory cells may represent the colon counterparts of Paneth cells. Other cell types include tuft cells, cup cells, and M cells, which reside on lymphoid Peyer's patches ([68], and refs. therein).

The role of the Wnt/β-catenin pathway as the crucial control of proliferation and differentiation of intestinal epithelial cells and so, of gut homeostasis is well documented. Wnt target gene expression indicates that the Wnt/β-catenin pathway is active in a gradient, with the highest activity at the crypt bottom. Wnt factors are secreted by pericryptal myofibroblasts and, in the small intestine, also by Paneth cells. The first indication of adult Wnt signalling controlling stem cell homeostasis came from a Tcf4 knockout experiment: mutant mice failed to build crypt stem cell compartments [71]. In concordance, transgenic expression of the Wnt inhibitor Dkk-1 [72, 73] or conditional depletion of β-catenin [74, 75] induced the complete loss of proliferating crypts in adult mice. Conversely, transgenic expression of the Wnt agonist R-Spondin-1 resulted in massive hyperproliferation of intestinal crypts [76]. More recently it has been shown that conditional deletion of Lgr4/Lgr5 in the mouse gut impairs Wnt target genes expression and results in a rapid destruction of intestinal crypts [77]. In conclusion, the Wnt/β-catenin pathway is essential for homeostasis of the intestinal crypt progenitor population, although specification of cell lineages depends on the crosstalk between Wnt/β-catenin and other pathways such as those of Notch, Hedgehog and bone morphogenetic protein (BMP) [78, 79].

4.3.3 Intestinal Stem Cells

Adult tissue stem cells are defined by longevity and multipotency, as they persist over long periods of time and are able to produce all cell types of the tissue to which they belong [68]. Two experimental strategies have been used to study and identify stem cells, transplantation and genetic lineage tracing [80, 81]. The transplantation approach utilizes molecular markers in order to enrich a tissue suspension in putative stem cell populations, followed by in vitro cell culture and transplantation into recipient animals. This approach has been highly successful in the identification of the hematopoietic stem cell from bone marrow [82], and cancer stem cells in leukemias [83] and solid tumors [84–87]. In the genetic lineage tracing approach stem cells are genetically marked in situ, and the introduced marker allows the visualization of the modified stem cell and its clonal offspring throughout time.

In the intestine the number of stem cells has been estimated to be between 4 and 6 per crypt. However, the precise location and identification of the ISC has for long been a matter of study and debate and two models coexist in the literature. The +4 position

model [88] proposes that ISCs reside directly above Paneth cells at the +4 position relative to the bottom of the crypt. These +4 cells are extremely radiation-sensitive, a property that would functionally protect the stem cell compartment from genetic damage. The stem cell zone model [89] was a consequence of the identification of a unique cell type populating the crypt bottom, which due to its morphology and location was named crypt base columnar (CBC) cell [90]. This population of small, cycling columnar cells is dispersed between Paneth cells. The recent discovery of Wnt target gene and R-spondin receptor Lrg5/Gpr49 as the specific marker for ISCs has allowed the identification of the CBC cells as the authentic ISCs [68, 91] and reinforces the tight bond between Wnt signalling and ISC homeostasis. Nevertheless, the existence of two populations of ISCs, quiescent and proliferating, and the sensitivity of ISCs to radiation and chemically-induced apoptosis are topics of intense discussion in current literature.

The Lgr5-GFP mouse model has been instrumental in the purification of $Lgr5^{+}$ stem cells from the small intestine [92]. These cells can grow in Matrigel 3D culture systems supplemented with a plethora of factors (EGF, Noggin, R-spondin, etc.) and form spheres with a central lumen and protruding buds. These experiments emphasized the central role of R-spondin for stem cell maintenance and demonstrated that complete organoids with a gut-like architecture and containing all epithelial cell types can be grown from a single $Lgr5^{+}$ stem cell. Batlle and cols. have been able to isolate and expand in vitro colon stem cells from normal human biopsies. By FACS, they purified crypt epithelial cells expressing epithelial cell adhesion molecule and high level of Ephrin type-B receptor 2 ($EpCAM^{+}$ $EPHB2^{high}$ cells). Although many isolated cells die, the surviving population grows to form spheroids in Matrigel that are similar to those observed in the mouse model [92]. These spheroids/organoids express stem cell markers such as Lgr5, Ascl2 and Olfm4, and differentiate in vitro into colon cell lineages when are cultured in the absence of prostaglandin (PG)E2 and Wnt3a and in the presence of a γ-secretase inhibitor that blocks Notch signalling [93]. Moreover, the establishment of culture conditions for ISCs allowed Yui and cols. [94] to expand Lgr5-GFP cells, which were subsequently engrafted into the damaged colons of immunodeficient ($rag2^{-/-}$) mice. These cells regenerated the damaged epithelium, restoring barrier function and giving raise to all differentiated lineages. Interestingly, the grafts were still contributing to epithelial homeostasis 25 weeks after transplantation while there were no signs of adenomatous or dysplastic changes. Therefore, the use of this ex-vivo expansion technology for regenerative medicine is an exciting and open issue. In summary, current data support that Lgr5 is an exquisite marker for stem cells at the bottom of the crypt, and the finding that Lgr5 is a receptor for R-spondins that potentiates Wnt receptor stability makes the relationship between Wnt signalling and ISC homeostasis come full circle.

4.3.4 Wnt/β-Catenin Pathway in Colon Cancer

Although Wnt signalling is essential to the normal physiology of the intestine, it was first characterized by its association with colorectal cancer, one of the most common cancers in western societies [95]. Colon cancer progression is thought to

be driven by an ordered sequence of mutations of which, invariably, the initiating mutation occurs in a gene (*APC*, *CTNNB1*/β-catenin, or *AXIN2*) that encodes a protein involved in the Wnt/β-catenin pathway. Loss of the tumor suppressor *APC* is the signature of the great majority of human intestinal tumors, both in the hereditary familial adenomatous polyposis and in sporadic colorectal cancers [96–98]. In the few cases where *APC* is not inactivated, human colon tumors arise from activating mutations in *CTNNB1*/β-catenin itself [99, 100], or from loss-of-function mutations in *AXIN2* [101]. As a common result, β-catenin accumulates in the nucleus, constitutively binds to LEF/TCF transcription factors and induces the expression of target genes mainly involved in cell proliferation, leading to the formation of benign yet long-lived adenomas. Subsequently, other mutations follow (e.g. in *K-RAS*, *SMAD4*, or *TP53*…), ultimately resulting in metastasizing carcinomas.

Transcriptomic studies revealed a specific gene program activated in *APC*-mutated human colon cancer that coincides with that expressed in crypts [102, 103]. Moreover, the population of tumor cells responsible for colorectal cancer initiation and progression, the cancer stem cells (CSC), displays elevated Wnt signalling as compared to more differentiated cells [104], and the Wnt target Lgr5 has recently been identified as a functional marker for CSC [105]. Batlle and cols. have shown that a gene signature specific for adult ISC predicts disease relapse in colorectal cancer patients. These ISC-specific genes identify a stem-like cell population located at the bottom of tumor structures reminiscent of crypts [106]. Strikingly and in apparent discrepancy, Medema's group has found that elevated expression of Wnt targets associates with good prognosis and that two of them, *LGR5* and *ASCL2*, become silenced by promoter methylation during tumorigenesis [107]. These authors conclude that methylation of Wnt target genes is a strong predictor of recurrence in colorectal cancer patients.

A modern concept that is gaining increasing acceptance is the crucial role of the tumor microenvironment (niche) for the generation and regulation of CSC and, in general, for tumor progression and metastasis. Tumor cells and CSC establish a mutual feed-back loop of exchange of signals with stromal cells, mainly myofibroblasts, smooth muscle cells, and recruited bone marrow precursors. Myofibroblasts secrete inflammatory cytokines that favor tumor progression and hepatocyte growth factor (HGF) that upon activation of its tyrosine kinase receptor c-MET, present in the plasma membrane of cancer cells, hyperactivates the Wnt/β-catenin pathway [104] (Fig. 4.2).

Further supporting a crucial role of Wnt/β-catenin signalling in colorectal cancer, sequencing of a large number of human tumors has confirmed that over 94 % harbour mutations in one or more components of the pathway [108].

4.3.5 Wnt/β-Catenin Pathway in Other Tissues

The crucial tumorigenic role of the Wnt/β-catenin pathway in the large majority of colon cancers cannot be extrapolated to other neoplasias. Abnormal activation of Wnt/β-catenin target genes has been detected in a variable proportion of liver cancer and in basically all cancers of the digestive tract, in which mutations in *APC*

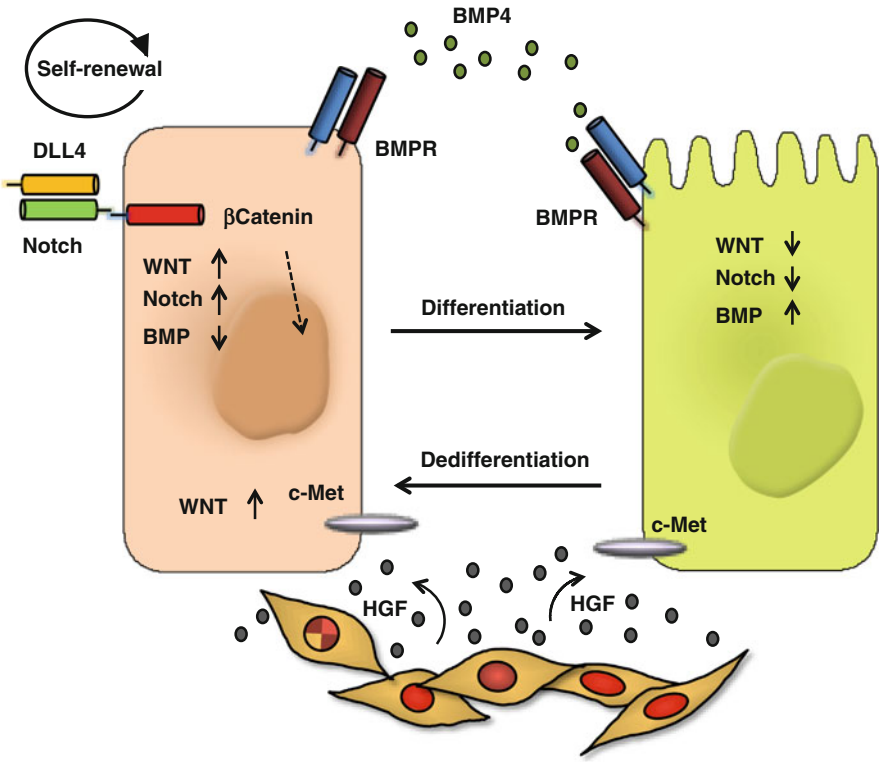

Fig. 4.2 Tumor microenvironment and colon cancer stem cells. Signals from the tumor microenvironment help to maintain colon CSCs. Wnt and Notch ligands promote self-renewal of CSCs while BMP4 antagonizes this self-renewal activity. Actually, BMP4 interferes Wnt signalling promoting differentiation. Colon CSCs in vivo are found in close proximity of HGF-producing myofibroblasts. HGF maintains colon CSCs in a stem-cell state and prevents differentiation. Moreover, HGF can activate the Wnt/β-catenin pathway in more differentiated tumor cells, reinstalling CSC characteristics (dedifferentiation) (Based on Medema and Vermeulen [79])

or *CTNNB1*/β-catenin genes exist. In breast cancer, accumulation of cytosolic and nuclear β-catenin is found also in a subset of tumors but many times in the absence of mutations in *APC* or *CTNNB1*/β-catenin genes. In these cases, the activation of the pathway has been explained by the local over-production of Wnt factors and/or the reduction in the level of inhibitors of the pathway (SFRPs, DKKs) [109]. Interestingly, activation of the Wnt/β-catenin pathway has been preferentially found in basal-like and triple-negative breast cancers, a type usually associated with the presence of high number of stem cells, poor outcome and lack of treatment [110, 111].

In line with its wide effects in many tissues during development, Wnt/β-catenin promotes differentiation of several types of cells (osteoblasts, neural precursors) and its role in several cancers such as medulloblastoma, melanoma or sarcomas is unclear, as in some cases the presence of active Wnt/β-catenin signalling appears to have anti-oncogenic effect or even correlates with good patient outcome [112, 113].

Remarkably, endogenous β-catenin is required for the induction of apoptosis of melanoma cells by the selective B-RAF^{V600E} inhibitor difluorophenyl-sulfonamine (PLX4720), a vemurafenib analog, and the activation of Wnt/β-catenin pathway synergizes with this agent to decrease tumor growth in vivo [114]. In addition, recent data suggest that Wnt/β-catenin signalling promotes aging-associated impairment of skeletal muscle regeneration [115].

4.4 Inhibition of Wnt/β-Catenin Pathway

The crucial importance of Wnt/β-catenin pathway in colorectal cancer and its contribution to several other neoplasias together with its role in the regulation of cancer stem cells make of it a highly interesting target of pharmacological intervention [116, 117]. However, the clinical use of Wnt/β-catenin inhibitors is delayed as compared to other signalling pathways. There are several reasons for this delay [118]. One is that, in contrast to other pathways, Wnt/β-catenin is not usually activated by a gain-of-function mutation. For instance, it lacks an oncogenic mutated kinase as potential druggable target. CK1 could be a suitable target as CK1γ and CK1ε phosphorylate LRP and Dvl, respectively; however, CK1α phosphorylates β-catenin favouring GSK3β action and so inactivates the pathway. Thus, CK1 isoform specific inhibitors would be necessary to effectively control the pathway. Another reason, which derives from the physiological function of Wnt/β-catenin pathway promoting differentiation of precursor cells in a number of tissues and organs (bone, brain…), is the potential toxicity of Wnt/β-catenin inhibitors. An additional possible toxicity is the interference with non-canonical Wnt pathways, whose implication in cancer and role in human physiology and development and in stem cell biology are not fully understood [67]. Paradoxically, the difficulties in inhibiting Wnt/β-catenin that will be reviewed below offer the possibility of a therapeutic window: it is plausible that a certain degree of inhibition of the pathway in adult life may cause acceptable toxicity (nowadays ignored in both qualitative and severity terms) while having beneficial effects in cancer patients in combination with other therapies.

A common problem with other signalling pathways is the existence of diverse manners in which Wnt/β-catenin pathway is activated in different neoplasias: mutation of *APC* or *CTNNB1*/β-catenin or *AXIN* gene in colon cancer, excess of Wnt factors, lack of Wnt inhibitors or abnormal splicing of LRP5 in breast cancer. Moreover, there seems to be a cross-talk between Wnt/β-catenin and other pathways (Notch, Hedgehog, TGF-β, HGF/MET) that is distinct in different types of cancer [119]. Also, although Wnt/β-catenin is a driver in colon cancer, when evaluating the effectiveness of Wnt inhibitors it has to be considered that these cancers contain at diagnosis important additional genes and pathways altered such as *TP53*, EGF, TGF-β and *RAS-BRAF*. Still, many studies in vitro and in animal models support the rationale and feasibility of the inhibition of Wnt/β-catenin pathway in colon cancer. As recently reviewed [116, 117], they are based on a variety of approaches that include the screening for compounds affecting the formation of β-catenin/TCF

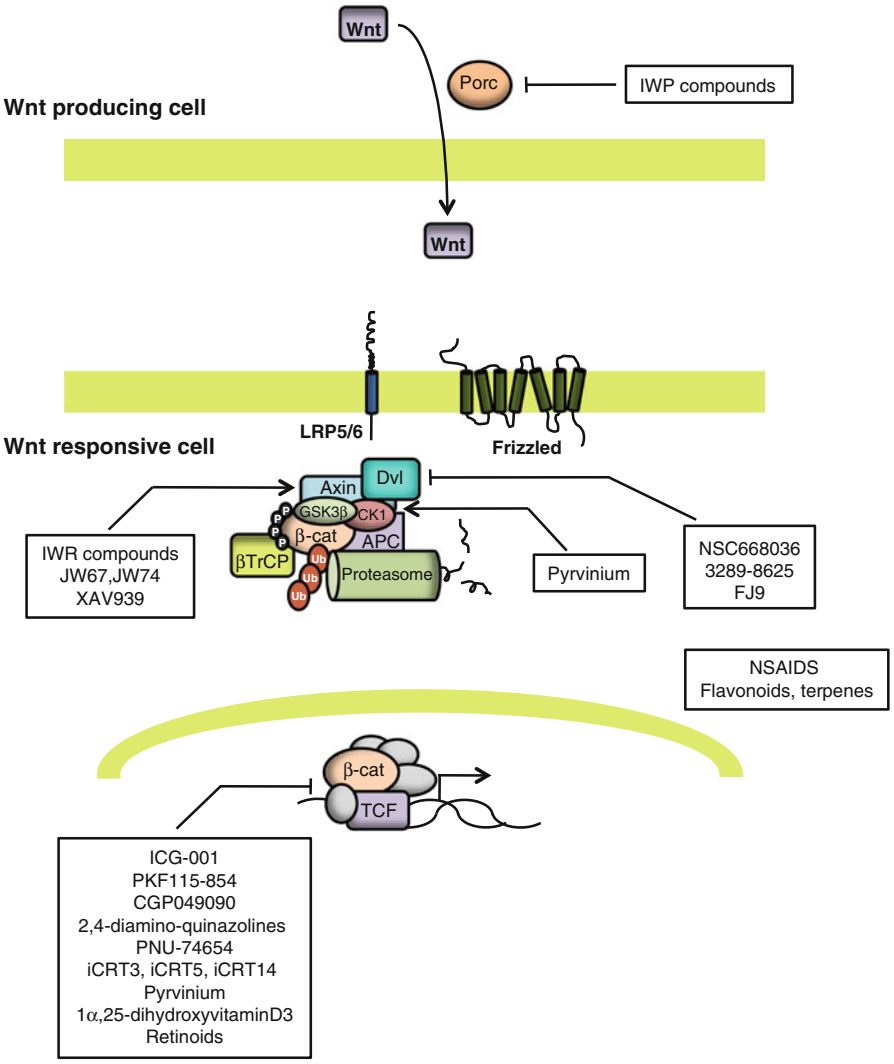

Fig. 4.3 Pharmacological inhibitors of Wnt signalling. Summary of current Wnt signalling inhibitors reported in the literature. Target protein(s) or processes interfered by the drug are indicated

complexes, downregulation of β-catenin by means of RNA interference or antisense oligonucleotides, forced β-catenin degradation by using several strategies, overexpression of an E-cadherin fragment to prevent nuclear translocation of β-catenin, knockdown of Wnt receptors, use of decoy Wnt receptors and antibodies, inhibition of Wnt factors secretion, ectopic expression of Wnt factors inhibitors (DKK-1, SFRP1, WIF1), Dvl antagonists or lytic viruses replicating selectively in cells with high β-catenin signalling, among others.

The brief history of the search for Wnt/β-catenin inhibitors may be summarized as follows (Fig. 4.3). Lepourcelet and cols. identified two natural compounds termed

CGP049090 and PKF115-854 that disrupted the β-catenin/TCF interaction [120]. These compounds inhibit the proliferation of cultured colon and hepatocellular cancer cells and induce apoptosis of leukemic cells [120–123], but no further development has been reported. Chen and cols. reported 2,4-diamino-quinazolines as inhibitors of the transcriptional activity of β-catenin/TCF complexes [124], while ICG-001 binds highly selectively the transcriptional co-activator CREB-binding protein (CBP) preventing its interaction with β-catenin and so, decreasing target gene expression [125, 126]. In silico virtual screening combined with nuclear magnetic resonance studies allowed to identify and synthesize molecules (FJ9, 3289-8625, NSC668036) that bind the PDZ domain of Dvl and inhibit the Wnt/β-catenin pathway ([127], and refs. therein). Also by combining virtual and biophysical screening Trosset and cols. identified PNU-74654 as a compound disrupting the interaction between β-catenin and TCF4 [128].

Thorne and cols. screened for compounds that both stabilize Axin and promote β-catenin turnover. They identified pyrvinium, an FDA-approved drug that inhibits Wnt signalling by binding to all CK1 family members [129]. Pyrvinium also promotes degradation of Pygopus, a component of the β-catenin/TCF transcriptional complex. Likewise, Chen et al. [130] reported several compounds as inhibitors of Wnt response (IWR) that stabilized Axin favouring β-catenin destruction. IWR-1 is an inhibitor of tankyrases, poly-ADP ribosylating enzymes that target Axin for degradation. The same authors described a second group of compounds termed inhibitors of Wnt production (IWP) that antagonized Wnt/β-catenin pathway by inhibiting Porcupine, the acyl transferase previously mentioned that is necessary for the modification and secretion of Wnt factors [130]. XAV939 is another tankyrase inhibitor described by Huang and cols. that works very nicely in vitro repressing β-catenin/TCF activity but has not reached the clinic [131]. JW67 and JW74 also stabilize Axin and inhibit β-catenin transcriptional activity via blockade of tankyrases 1 and 2 [132, 133]. Using a sophisticated screening, Gonsalves and cols. have identified three compounds (iCRT3, iCRT5, iCRT14) that antagonize β-catenin nuclear activity working downstream its destruction complex and so, without affecting the interaction of β-catenin with E-cadherin at the plasma membrane junctional structures [134].

A series of natural compounds, dietary agents or their derivatives, have been described as inhibitors of the Wnt/β-catenin pathway. Among them, several plant polyphenols (flavonoids) and terpenes such as genistein, quercetin, epigallocatechin-3-gallate, curcumin (diferuloylmethane), lupeol, lycopene and resveratrol [135, 136]. However, common characteristics of these agents are their unspecific or unclear mechanism of action and the usual high concentration needed to inhibit the Wnt/β-catenin pathway.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) such as aspirin, indomethacin, diclofenac or sulindac and several derivatives characterized as inhibitors of cyclooxygenases have also shown to decrease β-catenin nuclear content and target genes expression [135]. An attractive mechanism of action of NSAIDs has been proposed by Castellone and cols. that links COX and Wnt pathways [137]. These authors showed that following binding of prostaglandin E2 to its plasma membrane G-protein-coupled receptor Axin is recruited to the Gαs subunit. Thus, the

destruction complex of β-catenin is disassembled and β-catenin accumulates and translocates into the cell nucleus. In this way, by decreasing PGE2 levels through COX inhibition, NSAIDs control inflammation-associated Wnt/β-catenin pathway activation. Conversely, Lu and cols. proposed a mechanism by which NSAIDs inhibit this pathway that is independent of COX inhibition but, instead, requires high expression level of the nuclear receptors peroxisome proliferator-activated receptor (PPAR)-γ and retinoid-X-receptor (RXR)-α [138].

Retinoids (vitamin A derivatives) and vitamin D compounds, ligands for two other nuclear receptors, RARs and VDR, respectively, have been shown to repress Wnt/β-catenin pathway. 1α,25-Dihydroxyvitamin D_3 (Calcitriol) is the most active vitamin D compound. 1α,25-Dihydroxyvitamin D_3 is synthesized in the organism by successive hydroxylations in the liver, kidney and several other tissues of vitamin D_3 (cholecalciferol) obtained from the diet or produced in the skin by action of solar radiation on 7-dehydrocholesterol. In 2001, our group described that 1α,25-dihydroxyvitamin D_3 inhibits Wnt/β-catenin pathway in colon cancer cells by two mechanisms: (a) the rapid induction of complexes between its receptor VDR and β-catenin in the nucleus that competes for the formation of β-catenin/TCF complexes and, (b) the promotion of β-catenin nuclear export that is linked to the induction of E-cadherin expression and leads to the formation of E-cadherin/β-catenin complexes at the plasma membrane *adherens junctions* [139]. Later, a third mechanism of Wnt/β-catenin repression by 1α,25-dihydroxyvitamin D_3 was reported: the induction of the expression of DKK-1 [140]. The antagonism of Wnt/β-catenin by 1α,25-dihydroxyvitamin D_3 and other vitamin D compounds has been further analyzed and confirmed in different cell systems by other groups [141–144]. Moreover, Kaler and cols. have shown that by blocking the activation of signal transducer and activator of transcription (STAT)1 in THP macrophages, 1α,25-dihydroxyvitamin D_3 reduces the secretion of interleukin (IL)1-β, which activates Wnt/β-catenin pathway in colon carcinoma cells via inhibition of GSK3β activity and subsequent stabilization and nuclear translocation of β-catenin [145]. Retinoids also inhibit the Wnt/β-catenin pathway but their specific mechanism(s) of action are less established. Induction of β-catenin degradation or of inhibitory proteins such as Disabled-2 and formation of RXR/β-catenin complexes have been proposed ([135, 146], and refs. therein).

Another therapeutic possibility to target the Wnt/β-catenin pathway is the modulation of any of its physiological regulators, preferentially those acting within the cell nucleus (ICAT, Chibby, NLK…) that would hypothetically cause reduced toxicity [147]. However, this type of action remains basically unexplored.

As said before, the interest in targeting Wnt/β-catenin signalling has increased upon the finding that it is one of the pathways driving the proliferation of several types of stem cells and so, putatively, of cancer stem cells [116, 148]. The role of Wnt/β-catenin in the control of proliferation and/or differentiation of normal and cancer stem cells of different tissues is not well established [149]. In the intestine, Clevers' group has shown that the maintenance of the stem phenotype of cells at the bottom of the crypts appears to rely on the gene program driven by β-catenin/TCF4 complexes [102], and Batlle's group that the same gene program is characteristic of

colon cancer stem cells [106]. However, a better understanding of the biology of tissue-specific normal and cancer stem cells is needed before the clinical utility and the potential toxicity of the Wnt/β-catenin inhibitors in the pipeline can be established.

The complexity and high number of alterations present in cancer cells explain the usual requirement of combined treatments to obtain clinical responses. At the molecular level, this is based on the necessity to target more than one of the signalling pathways that are altered. However, the crosstalk between pathways has raised some unexpected results in terms of antagonism or dependence when using multiple inhibitors. An extreme case that implies Wnt/β-catenin is the recent description that high nuclear level of β-catenin may confer resistance to PI3K and AKT inhibitors and subvert the pro-apoptotic action of FOXO3a to promote metastasis in colon cancer patients [150]. This result strongly supports the necessity of a rational selection of patients to be subjected to targeted therapy and in particular the convenience of combined treatment with inhibitors of Wnt/β-catenin and PI3K/AKT pathways when colon cancer cells express concomitant high nuclear levels of β-catenin and FOXO3a.

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Chapter 5
Breast Cancer Stem Cells

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Abstract Cancer of the breast is the most common tumor in women in the Western-world countries. The expressions of luminal markers as ERα and GATA3 in luminal tumors suggest that their origin is normal luminal cells. Analogously, the expression of CK5 and CK17 in basal like tumors may suggest a source from myoepithelial/basal compartment. However this logic not explained as luminal progenitors are the target population for the development of basal-tumors. This finding supports the idea that the features of tumor not necessarily reflect the cell of origin. In this sense it would be possible that breast cancer stem cells (BCSCs) could play an important role in the origin of breast cancer. The Cancer Stem Cells Hypothesis has become more widely accepted. The existence of CSC in breast cancer has important consequences in prevention and therapy. Preventive strategies focused in reducing the number of CSCs as a mean to reduce breast cancer risk is a topic of high concern. The technological advances in CSC identification have favoured a better understanding of the possible mechanisms involved in the metastasis development and late relapse in breast cancer.

Keywords Breast cancer • Hedgehog • Inhibitors • Notch • Stem cells • Wnt

5.1 Cancer Stem Cells

Stem cells (SC) are essential participants in the normal physiology of the different tissues. These cells have three distinctive properties; the self-renewal, the capacity to generate multiple lineages and the ability to sustained proliferation. Cancer stem

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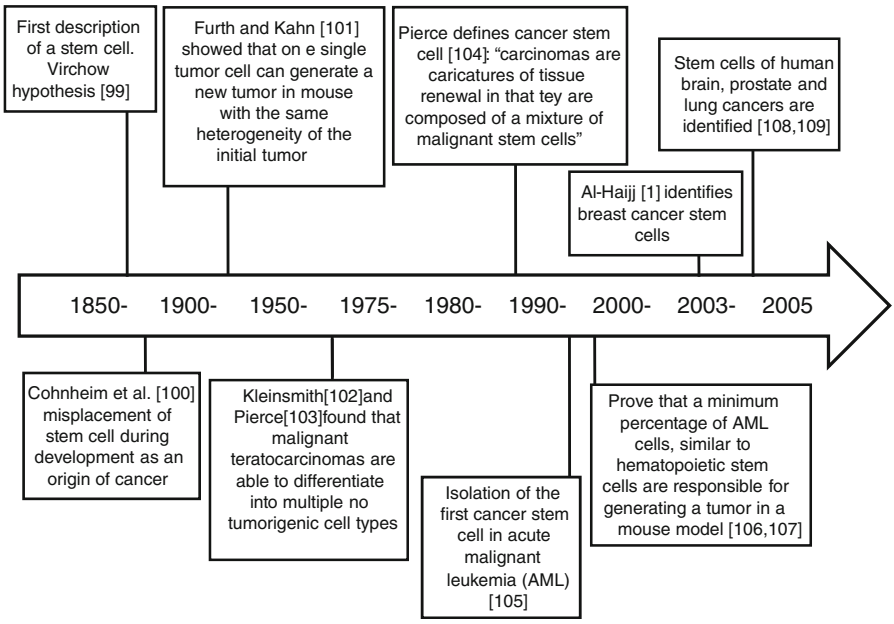

Fig. 5.1 Time line depicting the most relevant discoveries related to stem cells [99–109]

cells (CSC) as stem cells have this capacities but harboring malignant characteristics. Several studies showed these cells as tumor initiating cells (TICs) or tumor-propagating cells (TPCs) [1–3]. It is important to remark that CSCs not always arise from SCs transformation, they may also originate from differentiate cells that acquire stem cell-like properties [4–6] (see Fig. 5.1).

The existence of SCs is a feasible explanation for the tumor heterogeneity beside the clonal evolution model. This latter postulated that all tumor cells are capable of generating a new tumor and that each clone independently adapts and evolves resulting in the heterogeneity described. By other hand, the small number of SCs in the tumor, acting as multipotent progenitors, would be able to generate a new tumor that recapitulates the features of the original. These theories were not mutually exclusive.

Moreover there is controversy about the cellular origin of the different molecular subtypes of breast cancer. The expressions of luminal markers as ERα and GATA3 in luminal tumors suggest that their origin is normal luminal cells. Analogously, the expression of CK5 and CK17 in basal like tumors may suggest a source from myoepithelial/basal compartment. However this logic not explained as luminal progenitors are the target population for the development of basal-tumors according to the experiments reported by Lim et al. [7]. This finding supports the idea that the features of tumor not necessarily reflect the cell of origin. In this sense it would be possible that breast cancer stem cells (BCSCs) could play an important role in the origin of breast cancer.

BCSCs share characteristics with normal mammary stem cells such as the capacity to divide asymmetrically to generate one stem cell (self-renewal) and one progenitor cell destined for terminal differentiation that can produce diverse cancer cells. Both, CSCs and SCs can generate mammospheres. The mammospheres are composed

of single cell floating in non-adherent culture. The ability of cancer cells to induce tumors in severe combined immunodeficiency disorder (SCID) mice correlates with the capacity of form mammospheres [8, 9]. However it is not clear if mammospheres are composed by BCSC or downstream progenitors that recover stem cell-like properties [9]. Between the cancer cell the CSC have an enhanced capacity for tumor generation forming as well the basis of both recurrence and metastasis [10].

5.2 Origin

The prevalent idea form long time was that the majority of cancer cells have the ability to extensively proliferate and generate metastasis. However recent studies reduce these capacities to a subset of tumor cells that share properties with stem cells, been known as cancer stem cells.

As noted above, several of the features of CSCs can be found in normal SCs as the self-renewal by asymmetrical cell division, the sustained proliferation and the ability to generate multiple cellular lineages. A cancer stem cell produces a tumorigenic CSC and one not tumorigenic cell that ultimately differentiate. This hierarchical organization is very similar to the normal tissue cells. That fact has led to postulate that the mutations needed to generate cancer stem cells could be minor. The origin of BCSC is controversial, both as to the sequence of events that occur as the starting cell.

Respect to the original cell which leads to the CSC two hypotheses are the more accepted:

First, that the breast cancer stem cells derive from the deregulation of normal process of mammary stem cells, specifically the self-renewal and differentiation pathways. The fact that BCSCs and breast stem cells (BSCs) are very similar supports this hypothesis. Furthermore, the long lifespans of BSC makes it highly susceptible to mutations and oncogenic transformations [1, 2, 9].

The second hypothesis postulate that the BCSCs came from epithelial–mesenchymal transition (EMT). The cells that have undergone EMT have similar characteristics and behavior that normal and cancer stem cells. These cells also have an increased capacity to form mammospheres suggesting a high tumorigenic phenotype [11]. TGFB pathway is responsible of induction of EMT in mammary cells. Their activation causes also a positive autocrine feedback loop accelerating the conversion. Also, exogenous expression of the proteins Snail or Twist conferred the CD44+/CD24−/low phenotype [11–13].

How stem cells give rise to cancer is also matter of discussion. Considering potential changes or processes that could take place several hypotheses emerge:

1. Loss of the regulation by the microenvironment. The stem cells microenvironment plays an essential role in the regulation of the cellular cycle. Stem cells in tissues other than itself can cause neoplastic processes in the context of chronic inflammation [14]. The fact of being outside their usual niche may facilitate loss of control to disappear inhibitory signals from the extracellular matrix. Moreover the culture of stem cells increased the genetic instability promoting spontaneous mutations [15, 16].

2. Loss of the asymmetric division. Asymmetric division gives self-renewal capacity of stem cells. The stem cell divides into two daughter cells of which only one is like the mother. The cancer stem cells would lose the apical-basal polarity axis and both daughters cells would be identical to the mother driving accumulation of stem cells that lead to malignant behavior [17, 18]. Studies in neuronal stem cell of *Drosophila melanogaster* support this hypothesis [19].
3. Cell fusion. This common physiological process of muscle cells, gametes and placental tissue [20] can take place also between normal somatic cells to generate tumor cells. Furthermore the fusion between cancer cells and somatic cells can generate hybrid cells with higher malignancy that original ones. However these fusions not always conduce to the generation of malignant cells as described [21, 22].
4. Horizontal gene transfer. It is a similar mechanism that the used by bacteria to transfer antibiotic resistance genes. Stem cells through its phagocytic capacity could introduced apoptotic bodies rescheduled their genes (e.g. by regulatory RNA of malignant cells) converting it into a tumor [23].

5.3 Markers

Breast cancer stem cells we initially discover for Al-Hajj et al. [1] when found a subpopulation from human breast cancer tumors with a highly tumorigenic phenotype. This population was characterized by the cell surface markers ESA+/CD44+/CD24–/low and CD140b (Lin-). A 100 cells with these characteristics are capable of generating a tumor in a mouse xenograft model. In contrast, 50,000 unselected tumor cells are required for the generation of tumors in these conditions. Furthermore, the tumors cells generate in the mouse by BCSCs recapitulate the molecular heterogeneity of the original population.

CD44 is a transmembrane glycoprotein that regulates the adhesion between cells, with the matrix as well as the cell migration. The gene encoding CD44 consists of 20 exons, 10 of them are transcribed in the standard form. Multiple isoforms arise from alternative splicing of other ten exons. The standard form is ubiquitous located in lymphocytes and epithelial cells. However the splicing variants have tissue specific expression. Specifically the variant CD44v6 have been associated with poor prognosis in cancer [24].

CD24 is a mucin-type protein heavily glycosylated and linked by a glycosylphosphatidylinositol (GPI) anchor to the cell membrane. This protein is involved in the regulation of the cell proliferation and cellular interactions. Furthermore is a ligand of the adhesion receptor P-selectin, suggesting a role in the metastasis process [25].

Subsequent studies have been successful to isolate BCSCs using this phenotype [26, 27]. However, some authors failed to confirm the association of CD44+/CD24–/low with clinical outcome in breast cancer [28] and other publications found that breast cancer with opposite phenotypic patron (CD44–/CD24+) associate with poor prognosis [29]. Campbell et al. proposed that both populations (CD44+/CD24–/low and CD44–/CD24+) competing for dominance in the clonal model [30].

The selection of CD44+/CD24–/low phenotype in combination with ALDH1+ enriches the tumorigenic capacity [2] of these cells. Similarly the epithelial cell adhesion molecule EpCAM was found to enrich the fraction CD44+/CD24–/low for CSCs [2].

It is important to note that not always the described markers identify BCSCs. The Al-Hajj's study were not able to successful identified the CSC of one patient by the CD44+ CD24−/low phenotype. Other authors have found that CD44+/CD24−/low and ALDH1 did not universally detected CSC [31]. It has been proposed that BCSCs similarly to hematological malignancies present heterogeneity between subtypes of breast cancers [32, 33]. Indeed, the CD44+/CD24−/low phenotype was established using only 9 metastatic breast cancer patients and need to be validated across a sufficient and representative number of breast cancer samples.

In this sense, the immunohistochemical analysis of the CD44+/CD24−/low phenotype reveals that is present only in the 31 % of breast cancer and show that the basal-like subtype presents the highest percentage versus other breast cancer subtypes. Also, this relation is evident in BRCA-1 inherited cancer. The expression of ALDH1 is not associated with a particular subtype. However, the patron CD44+/CD24−/low ALDH1 phenotype also correlates with basal like subtype [34, 35] and has been associated with more aggressive tumor phenotype [9]. CK18, GATA3 and MUC1 expression is more frequent in luminal subtypes [36]. However, studies have shown that these markers varied among samples of patients with different molecular and clinical features [37].

Another potential stem cell marker is CD133 (prominin-1) located in the membrane protrusions. It has been found in the bone marrow, in different tissues and solid tumors, but in the latter with expression limited compared to CD44 + and ALDH1. Expression of CD133 has been reported in inflammatory and triple negative breast cancers [38, 39].

Other authors used CD29 (β1-integrin), CD49f (α6-integrin) [40] and CD61 (β3-integrin) [41] as stem cells markers.

This data highlight the importance to identify the adequate BCSCs combination of markers for each subtype of breast cancer.

Additionally to immunolabeling o flow cytometry analysis with cellular markers other assays have been used to identify or isolate breast cancer stem cells. The ALDEFLUOR assay measures the ALDH1 activity. The "side population assay" used the lipophilic fluorescent dye Hoechst 33342 to evaluate the capacity of cells to efflux this compound by a mechanism similar to the expulsion of drugs [42]. Finally, other functional assay is the mammosphere formation assay. Mammary epithelial cells are cultured without serum on a not adherent surface, and accordingly are forced to form three-dimensional clusters called mammospheres [9]. The capacity of form floating spherical colonies correlates with the ability to induce tumor in SCID mice [8, 9]. Figure 5.2 shows the different methods to detect stem cells.

5.4 Pathways

CSCs are capable of self-renew and differentiation expressing activation of the cell signaling pathways of normal stem cells.

In mammary stem cells Hedgehog, Wnt and Notch signaling pathways are the main responsible for self-renewal. BCSCs share cellular marker and pathways with normal stem cells but with an aberrant activation that may lead to cancer. The sequences of events that lead CSCs to acquire their characteristics vary between

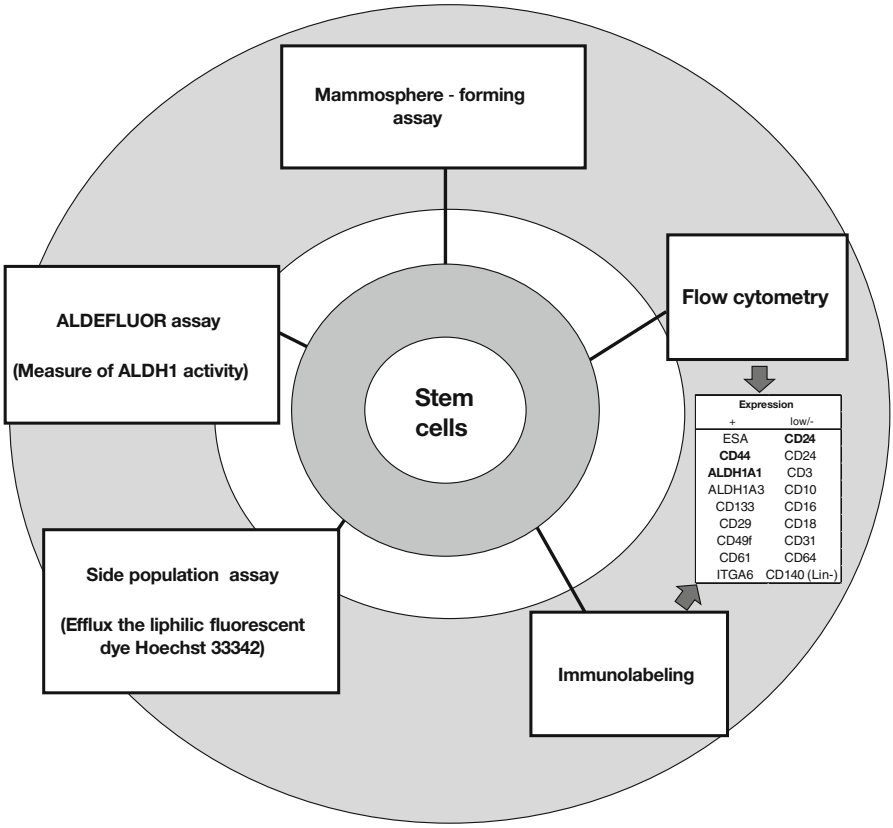

Fig. 5.2 Representation of the most relevant methods for detecting stem cells

tumors. It is important to discern which mechanisms are involved in each case. We describe the main pathways in CSCs, focusing in breast cancer.

5.4.1 Notch Pathway

The conserved Notch pathway is involved in cell fate, differentiation and proliferation. It inhibitory or inductor role is highly dependent of the cellular context. This pathway is composed by four receptors (Notch1, Notch2, Notch3 and Notch4) that were transmembrane proteins. The five ligands: delta-like proteins (DLL1, DLL3, and DLL4) and Jagged proteins (JAG1 and JAG2) are also transmembrane proteins and the activation occurs by direct cell-cell contact. This activation triggers to consecutive cleavage by the protease ADAM and the γ-secretase that release the active intracellular domain. This domain translocate to the nucleus, binds to the CSL (CBF1/Suppressor of Hairless/LAG1)/RBPJ transcription factor activating the transcription of genes as PI3K,AKT, PPAR, CyclinD1 and NFkB.

In the context of the mammary gland and particularly in the stem cells the Notch pathway is important for self-renewal, proliferation and the regulation of cell fate [43]. Notch is involved in the commitment of bipotent cells into the myoepithelial lineage and may also have a role in the later differentiation of luminal progenitors. Indeed, the expression of Notch-1 is increased during luminal differentiation and it is specifically expressed in this cell subtype. Moreover, it has been proposed that Notch- 4 regulate the transition from stem cell to progenitor cell.

The activation of this pathway is common in breast cancer been detected in 50 % of the cases [44]. Nocth-1 and Notch-4 are involved in the normal development of the mammary gland and it is believed that mutations in these receptors are early event in breast cancer since these are detected in ductal carcinoma in situ.

Overexpression of the Noth pathway has been related to chemoresistance [45] and radioresistance [46]. This effect may be mediated, at least in part, by the ability to induce cyclin D1 and the antiapoptotic gene BIRC5 (surviving) [47]. Cyclin D1 is required for self-renewal and is capable to increase the Notch1 activity by inhibition of its negative regulator Numb [48]. Cyclin D1 may be an important target as downstream effector of several pathways as Wnt, NFkB, Stat3 and β-catenin [49].

Notch-4 is important for self-renewal and elevated expression of Notch-1 and the receptor JAG1 has been related to poor survival [44, 50]. A decreased level of Notch-1 induces apoptosis and sensitizes tumors to doxorubicin supporting it role in cell growth. The expression of Notch-3 also has been related to tumorogenesis [51].

Notch signaling factors have been up-regulated in CSCs. The activation of this pathway enhances mammospheres formation and it has been suggested a role in lineage-specific differentiation. Instead, the blockage of Notch pathway reduces the mammosphere formation, the frequency of CD44+/CD24−/low cells and the ability to form tumor in vivo[52].

The Notch-1 activation is able to transform mouse epithelial cells in vitro, while the activation of Notch-4 inhibits the differentiation of epithelial cells. However, only the second is able to generate mammary adenocarcinoma in mouse [44].

The available data suggest that deregulated Notch signaling may contribute to tumor development by deregulation of normal stem cell activity [44]. However, there exist controversy because in contrast to the studies that show activation of this pathway in SC, others publications relate the down-regulation of Notch signaling with elevated reconstitution of the mammary stem cells [37].

5.4.2 Wnt Pathway

Other important pathway in BCSCs is Wnt. This complex pathway is composed of 19 ligands and several receptors. It activation give expansion of mammary stem cells [27, 28].

Studies of Wnt-1 activation in mouse model suggest that plays a role in tumor initiation [53, 54]. Some authors found that the Wnt signal inhibits the factor de transcription GATA3 that promote differentiation into luminal cells. Based on this

the proposal is that Wnt could prevent differentiation and thereby leads to self-renewal.

By other hand, the activated β-catenin is sufficient to induce mammary tumor initiation [55]. However mutations and deregulation of Wnt pathway are not common in breast cancer unlike other types of tumors as colon.

Although mutations were not common, in breast cancer have been detected upregulations of Wnt activity with expression of β-catenin in 60 % of the tumors, elevated levels of CCND1 (target of β-catenin/TCF/LEF transcriptonial activation), decreased expression of the Wnt inhibitor factor (WIF1) and elevated expression of Wnt ligands [53, 56]. Also aberrant methylation of APC promotor has been described in breast cancer. Those modifications correlated with a poor prognosis in patients.

It is possible that other signaling pathways impinge on Wnt leading to hyperactivity of the pathway, for example by EGF or GSK3B. Furthermore, two factor commonly loss in cancer, p53 and PTEN, negatively regulate β-catenin.

Finally, altered Wnt signaling have related to high level of basal markers driving the hypothesis of a Wnt as inductor of dedifferentiation of mammary cells. Also, Wnt have shown a predictive role for brain metastases [29].

5.4.3 Hedgehog Pathway

The third relevant pathway in BCSCs signaling is Hedgehog, necessary for normal development of the mammary tissue [57]. Hedgehog pathway is activated in BCSCs and inhibition of its signal result in a reduction of the mammospheres formation.

Hedgehog signaling pathway regulates embryogenesis, morphogenesis, proliferation and differentiation and it's responsible for CSCs maintenance. Moreover it plays a critical role in epithelial-mesenchymal transition (EMT) process which is necessary for tumor invasion and metastasis.

The hedgehog (Hh) ligands bind to a 12-pass transmembrane protein Patched (PTCH) and these binding results in a depression of Smoothened (SMO). SMO then translocates to the primary cilium which is internalized and activated. Then transcription factors GLI-1, -2 and -3 are activated leading to transcription of GLI target genes. The balance between the activator and repressor forms of GLI factors moderates Hh signaling [58].

Data from many human tumors including breast cancer have suggested that Hh signaling plays an important role in CSC regulation [59]. CSC is defined by their capacity to self-renewal and differentiation that recapitulates the original tumor in an ectopic environment. Self-renewal is crucial for the maintenance of the malignant clone and previous studies have provided evidence that Hh signaling regulates this issue [60]. In breast cancer, pathway activation in CSCs using Hh ligand and GLI1 or GLI2 alters the expression of BMI-1, a central regulator of self-renewal in normal stem cells [59].

These observations suggest an important role of Hh pathway in the regulation of self-renewal properties of the CSC.

5.5 Biological Implications

5.5.1 Breast Cancer Risk and SC

The cancer stem-cell hypothesis proposes that cancers arise in breast through deregulation of the normally regulated process for self-renewal. If breast tumors originate in mammary SCs then breast SCs number may be a risk factor for carcinogenesis.

Several studies have shown a relation between birth weight and breast cancer risk, as well as maternal levels of insulin-like growth factor (IGF-1) and birth weight [61]. The growth hormone/IGF-1 axis may play a role as regulator of CSCs in different organs. Mammary stem cells may overexpress growth hormone receptors and previous data suggest that growth hormone may stimulates CSC self-renewal suggesting a potential link between breast cancer risk in high birth weight and CSC regulation [62].

5.6 Clinical Implications

5.6.1 Breast Cancer Prevention and CSCs

Prevention effects of dietary modifications modulating stem-cell number during key developmental windows in utero and adolescence have been suggested by animal models [62]. In this context, agents such as phytoestrogens or curcumin may modulate SC self-renewal pathways such as Wnt and Notch [63–65]. Preclinical studies have confirmed that the dietary polyphenols curcumin and piperine inhibit breast cancer and normal SC self-renewal but cause no toxicity to normally differentiated mammary cells, suggesting a potential role as cancer preventive agents [66].

Vitamin D3 has also been shown to be involved in SC differentiation and therefore may have applications for cancer prevention strategies targeting SC [67].

5.6.2 Metastasis

There is increasing evidence that cancer stem cells play an important role in mediating tumor metastasis. As described previously, breast cancer stem cells have been characterized as having the cell-surface phenotype CD44+/CD24–/low. CD44 is a cell adhesion molecule involved in binding of cells to hyaluronic acid, whereas CD24 is a negative regulator of the chemokine receptor CXCR4, a molecule involved in breast cancer metastasis [28]. To determine the relationship of the CSC phenotype to metastasis, Balic et al. [68] examined the expression of CSC markers in metastatic bone marrow in patients with breast carcinoma

and found an increased in CD44+/CD24−/low expressing cells. Although the presence of micrometastasis is associated with poor prognosis [69], approximately 50 % of patients with such metastasis do not develop clinically apparent macrometastasis within a 10-year follow up period. Several studies have tried to determine whether expression of SC markers in bone marrow and lymph node metastasis may predict relapse.

5.6.3 Tumor Dormancy and Relapse

During metastatic dissemination process, primary tumors shed millions of cells into the blood however only few of them will lead to secondary tumors [70]. Therefore metastatic growth will be only achieved if the metastatic cell has self-renewal and tumor-maintenance capacity [71].

Metastatic tumour cells usually carry genetic or epigenetic changes that enable motile and invasive properties acquiring the capacity of degrading the basement membrane and invading the underlying stroma. The invading tumor cells interact with fibroblast or immune cells of the stromal matrix. As a consequence of this cross-talk tumor cell-stromal cell the extracellular matrix and the vascular walls are degraded. Breast cancer tumor cells can arrest in lymph nodes, bone marrow or in the target organ vasculature where they can extravasate into the organ parenchyma. At this stage tumor cells have four possibilities: they die (the majority of cells initiate apoptotic mechanisms), they can enter a state of quiescence or dormancy either as solitary tumour cell or as a micrometastatic lesion that underwent a proliferation expansion and cannot recruit a vascular bed or they can resume proliferation becoming a growing micrometastasis [72].

In the last years increasing evidence suggest a parallelism between tumor dormancy and the CSC theory of tumor propagation [73]. It is not unfrequent that breast cancer relapses occurs after many years after resection of primary tumour. Bone marrow micrometastases have been identified in patients diagnosed with breast cancer at early stage as an adverse prognostic factor for recurrence [69]. Reactivation of a previously dormant pluripotential cell located in bone marrow or lymph nodes could explain these long-term relapses. In this context a recent study identified a higher percentage of cells CD44+/CD24−/low in bone marrow of patients with high risk clinicopathological features [74].

Mechanisms that activate a previous quiescent cell leading to a potentially metastasis-generating cell are not well known. Immune environment modifications [75, 76], or a disbalance between pro and anti-angiogenic factors, a phenomenon called angiogenic switch [77], have been postulated as possibly responsible for this change.

It has been suggested that the cancer initiation clone is originated from CSCs as only SC live long enough to develop tumors after long period of time.

5.7 Treatment

Given the fact that CSCs have been associated to metastasis, tumor progression and dormancy it is reasonable to suppose that therapeutic efforts should be focused on stem cells. The cancer stem cell hypothesis suggests that those strategies targeting breast stem-cells population must be effective for breast cancer treatment and prevention.

5.7.1 Response to Chemotherapy

Several preclinical studies conducted in breast cancer mammary models have showed survival or significant enrichment of CD44+/CD24−/low cell after chemotherapy administration [78] suggesting a primary resistance of CSC to chemotherapy.

An in vitro study in breast cancer cell line confirmed that after therapy with paclitaxel and epirubicin the vast majority of surviving cells expressed the phenotype CD44+/CD24−/low [79].

The role of CSC in the response to chemotherapy has been widely studied in HER2+ breast cancer. Trastuzumab is a monoclonal antibody that blocks HER2 signalling and remains the mainstay of the treatment in HER2+ breast cancer. But trastuzumab mechanism of action is not only limited to a direct HER2 receptor-trastuzumab interaction. Instead, trastuzumab also recruits cytotoxic effector cells via the Fcg-part of this IGg1 antibody and thus induces an immune response effected by granulocytes, monocytes, macrophages, dendritic cells and natural killer (NK) cells named "antibody-dependent cell mediated cytotoxicity" (ADCC). Preclinical experiments further showed that trastuzumab-induced ADCC depends on the Fc-part of the antibody, the availability of FcgRIIIA on NK cells and the presence of interleukin 2 suggesting a significant role of NK cells.

In 2009, Reim et al. [80] studied the role of ADCC mediated by trastuzumab in breast cancer cell lines. An in vitro immunoselection of ADCC breast cancer cell lines was performed by previous exposure of these lines to trastuzumab and polyclonal NK cells. The MCF-7 cell line failed to develop immunoresistance in vitro, instead, these cells displayed a CD44+/CD24−/low phenotype and showed a reduced overexpression of HER2 mimicking the CSCs phenotype. When the immuneselected cell population was re-expanded, cells lost the CSCs phenotype and displayed the initial HER2 surface expression. These findings support the clinical observation that trastuzumab might be effective even beyond progression.

However, other studies have identified that CD44+/CD24−/low cells might be involved in mechanisms of the novo resistance to trastuzumab therapy [81]. Latter studies have confirmed the importance of CSCs in the resistance to trastuzumab and have suggest that treatment with metformin might synergistically interact with

trastuzumab leading to suppress self-renewal of CSCs/progenitor cells in HER2+ breast cancer lines [82].

More recent in vitro results suggest that the combination of trastuzumab and pertuzumab targets more efficiently a subset of CD44+/CD24−/low/HER2 low cells than any single antibody. The authors consider that the ability of these antibodies to recruit natural killer cells and subsequent induct of antibody-dependent cell-mediated cytotoxicity might be responsible for this effect [83]. These data suggest that CSCs might be treated not only by a directed effect of the targeted compounds but by immunotherapeutic effects.

Recent data indicate however, that CSCs act as a subpopulation of drug resistant cells that survive chemotherapy and have the potential to repopulate the tumor. Stem cells chemoresistance might be explained by a slow proliferation in the G0 phase of the cell cycle making they therefore resistant to cell-cycle active drugs [10]. Additionally, resistance to apoptosis due to expression of antiapoptotic proteins such as Bcl-2 [84] or expression of high levels of multifunctional efflux ATP dependent transporters linked to multidrug resistance might be other contributing factors.

Taking together these data underline the importance of CSC in drug resistance to chemotherapy and anti-HER2 agents.

5.7.2 Response to Endocrine Therapy

Several data support the hypothesis that CSC may play a crucial role in resistance to endocrine therapy. Recently it has been identified a subpopulation of tumor cells negative for estrogen (ER) and progesterone receptors (PR) and expressing CD44+/CK5+ that shares self-renewal properties with CSC. Exposure to anti-estrogen therapies such as tamoxifen or fulvestrant lead to a selective enrichment with these cells whereas the sensitive population of ER+/PR + breast cancer cells decreased. It has been postulated that this population of ER-/PR-/CD44+/CK5+ might play a role in the acquired resistance to endocrine therapy in hormonesensitive breast cancer [85, 86].

5.7.3 Targeting CSCs

Theoretically if CSCs were deleted, the remaining cells would be unable to re-growth and promote to a new tumor. This concept has led to develop several attempts to target self-renewal or regulatory pathways of the CSCs. However most of these strategies are in a preclinical phase and are based on in vitro observations, only a few of these inhibitors have started their clinical development in several trials for various diseases [87]. The first approach targets the specific surface markers. Monoclonal antibodies targeting CD44 have shown reduction of tumor growth in xenographs [88]. Similarly targeting the ALDH1 marker with CD38-T cells eliminates CSC inhibiting tumor growth [89].

Other strategies against CSC specifically inhibit transduction pathways. Notch pathway is crucial for CSC self-renewal and for normal breast development. Some data suggest that Notch inhibition could be used as chemoprevention for ductal in situ carcinoma [90]. Furthermore Notch inhibition in cell lines has led to elimination of CSC and arrest of tumor growth [52, 91]. Inhibition of Notch pathway has been tested with specific secretase inhibitors, genomic inhibition with short hairpin RNA (shRNA), immunotherapy or monoclonal antibodies against membrane Notch receptors.

Notch signalling is activated in HER2+ breast cancer lines. Specific Notch inhibition reduces HER2 cell expression in xenografts [92] suggesting that Notch inhibition may be an interesting strategy to overcome potential resistance to HER2 pathway inhibition.

Recent studies in triple negative cell lines showed that Notch inhibition with specific monoclonal antibodies lead to tumor growth inhibition, particularly when administered with docetaxel. Anti-Notch monoclonal antibodies caused a reduction of the mammospheres formation of CD44+/CD24−/low cells. Moreover this strategy resulted in tumor incidence after re-implantation and delayed tumor recurrence [93].

Another signalling pathway possibly involved in CSC regulation is PI3K-AKT-mTOR and particularly through the activation of STAT3. Previous data showed that inhibiting the JAK2/STAT3 pathway targeted the population of CD44+/CD24−/low cells in basal-like breast cancer cell lines leading to decreased tumor growth in xenograph models [94]. Furthermore a previous study in vitro showed that AKT inhibition lead to a decrease in the number of CSC [95].

Inhibition of Wnt signalling by dietary curcumin has been shown to decrease CSC with no toxicity on normally mammary cells. A preclinical study in cells lines and animal models showed that Wnt signalling is effectively disrupted through the inhibition of porcupine, a membrane bound O-acyltransferase. Surprisingly, in mice, Wnt inhibition exhibited no toxicity and at therapeutic effective dose there were no pathologic changes in gut or in other tissues [96]. These findings suggest that Wnt pathway inhibition, apart from a potential preventive strategy to minimize breast cancer risk, could be an attractive strategy in combination with chemotherapy.

But apart from specific inhibitors targeting the most relevant signalling pathways related to self-renewal, several drugs developed for other diseases have shown to be effective against CSC. One example for these compounds is metformin. Metformin, an antidiabetic oral drug, has shown to decrease the number of breast CSCs in vitro [97]. Of note, combined administration of metformin and doxorubicin in cell cultures eradicated both CSC and non-CSC tumor cells, whereas treatment with doxorubicin in monotherapy was ineffective for eliminating CSC.

As previously mentioned, several studies have suggested that CSCs play an important role in the resistance to trastuzumab. In vitro experiments showed that metformin synergistically interacts with trastuzumab leading to suppress self-renewal of CSCs/progenitor cells in HER2+ breast cancer lines [82]. Moreover, metformin overcame primary resistance to trastuzumab in an in vivo experiment with a HER2+ breast cancer cell line obtained from a pleural metastasis of a patient with *de novo* resistance to trastuzumab. Addition of metformin to trastuzumab in this cell line induced a sharp reduction of the tumor in comparison to exposure to

trastuzumab monotherapy. This data suggests that targeting CSC with metformin could be an effective strategy for overcoming resistance to trastuzumab [98].

Finally, other potential target for breast cancer CSCs is c-KIT as it has been identified in luminal progenitor cells [7] however these findings should be considered with caution, as c-KIT is strongly expressed by normal breast epithelium.

Despite the availability of several inhibitors in the therapeutic arsenal, targeting CSCs is not easy, as their plasticity and genomic instability allow these cells to evade the treatment. Moreover the cross-talk among different pathways might provide many mechanisms of resistance. Although preclinical data are encouraging, these effects must be confirmed in prospective clinical trials.

5.8 Conclusions and Future Perspectives

The Cancer Stem Cells Hypothesis has become more widely accepted. The existence of CSC in breast cancer has important consequences in prevention and therapy. Preventive strategies focused in reducing the number of CSCs as a mean to reduce breast cancer risk is a topic of high concern.

In the therapeutic arena, a better knowledge of the biology of CSC may lead to identify new targets for personalized therapy. Moreover, CSC seems to play a role in the development of resistance to many several conventional drugs of our actual arsenal, such as chemotherapeutic, endocrine or anti-HER2 agents. An appropriate knowledge of CSC mechanisms will allow establishing new strategies to overcome resistance to conventional therapies.

Finally, the technological advances in CSC identification have favoured a better understanding of the possible mechanisms involved in the metastasis development and late relapse in breast cancer.

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Chapter 6
Stem Cells in Colon Cancer

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Abstract Colorectal cancer (CRC) is still one of the most commonly diagnosed and lethal cancers worldwide. The classic model of CRC carcinogenesis involves a multistep process of oncogenes activation and inactivation of tumor suppressor genes. But the cell of origin and the type of cells that propagate the tumor after its initiation are still unknown.

The concept of Cancer Stem Cells (CSC) was first developed for hematologic malignancies and later applied to solid neoplasias. This model suggests that tumors are hierarchically organized and only CSCs possess the ability to initiate tumors and due to their resistance to conventional treatments, they are also responsible for tumor relapse. The problem lies still in their identification which remains controversial due to the lack of specific molecular markers.

Colon CSCs were originally identified through the expression of the CD133. However, it is not definitively proven that CD133 is a reliable marker of colon CSCs and other cell surface markers, such as CD44, CD166, Musashi-1, CD24 among others have also been suggested.

Moreover, there are several molecular pathways (Wnt or Notch) as well as the complex crosstalk network between microenvironment and CSCs which are relevant for CRC.

Therefore the design of CSC-targeted agents would enhance responsiveness to traditional treatments and eventually reduce local recurrence and metastasis.

This review will discuss the newly introduced CSC model in CRC, the identification markers and the pathways involved in the design of novel therapeutic approaches and also the limitations associated with this model.

Keywords Colon cancer • Crypt • Stem cells • Transforming growth factor b • Wnt

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6.1 Introduction

All different organs in human organism are constituted by tissues with specialized cells and their specific stem cells which represent only a small fraction of the tissue but with capacities of self-renewal and differentiation into mature cells which aim at maintaining the tissue integrity [84].

There are consistent relationship between deregulation of stem cells and carcinogenesis. In fact there are regulatory mechanisms of self-renewal in normal stem cells that also regulate oncogenesis. In this way recent experimental and clinical evidences support the hypothesis that cancer may arise from mutations in normal stem cell populations, and thereafter these cells will suffer from ongoing genetic and epigenetic changes that could contribute to settle the disease [67].

This is what *the stem cell model* proposed by Wang and Dick considered. Each cell has a different probability of acquisition of a specific tumoral phenotype and thus, tumors are more likely considered as a complex tissue where tumor initiation and growth is led by a minority of cells called "tumor-driving cells" or "cancer stem cells" (CSCs) [6, 101].

In fact, there are several investigations that recently have identified specific CSCs markers showing similar expression profiles than the normal stem cells of the same organ. In addition, CSCs can be prospectively isolated based on the expression of these molecules and have the capacity to develop tumors when xenografted in immunodeficient mice [67].

There are even more evidences that normal stem cells can play a role in carcinogenesis. The similarities found between normal adult stem cells and cancer cells such as self-renewal capacity, the production of differentiated cells, activation of antiapoptotic pathways, induction of angiogenesis, resistance to apoptosis and drugs due to active telomerase expression and elevated membrane transporter activity, and finally the ability to migrate and propagate [103]. But as opposed to normal adult stem cells that remain constant in number, CSCs can increase as the tumors grow and give rise to a progeny that can be both locally invasive and/or metastasize. Thus CSCs knowledge has become a key in finding the target population of tumoral cells for therapeutical advances.

Although as these tumor-initiating cells have been identified in hematological malignancies, there are still controversial observation in solid tumors which require further research [37].

One of the most frequent cancers in the world is colorectal cancer (CRC) and despite all the advances achieved in its treatment, it is still a leading cause of morbidity and mortality worldwide with a cumulative incidence rate of 9.4 % [47].

The progression from adenoma to localized cancer and then metastatic disease require the simultaneous failure of protective mechanisms such as adenomatous polyposis coli (APC), p53, and transforming growth factor b (TGF-b) and the induction of oncogenic pathways, such as Ras [52, 56].

Although traditional models of tumorigenesis (as the stochastic model) suggest that every tumoral cell is able to initiate a tumor, the newly proposed CSCs model considers that only a small fraction of cells possesses tumor generation and propagation abilities [44].

This hypothesis raises questions regarding the efficacy of current therapies and suggest that CSCs are a rational target for the development of firm therapeutic strategies.

6.2 Intestinal Organization and Normal Colon Stem Cells

The mammalian intestinal epithelial wall faces a harsh luminal environment and it is critical to digest and absorb the nutrients and thus it requires constant renewal. This process involves rapid and continuous proliferation of epithelial cells in the crypt base with ulterior migration of these cells to the luminal surface. All this process seems to be completely dependent upon a limited number of long-lived multipotent intestinal progenitor cells or stem cells [33].

The large mammalian intestine is divided into four distinct anatomical layers. The epithelial layer at the luminal surface is formed by a single sheet of epithelial cells folded into finger-like invaginations. This layer is embedded in the submucosal connective tissue to constitute the functional unit of the intestine called the crypt of Lieberkühn [81].

In the adult human colon there are approximately 14,000 crypts per square centimeter and each crypt contains approximately 2,000 cells [11, 17, 73–75] and comprises three main differentiated types of cells: colonocytes or absorptive enterocytes, mucus-secreting goblet cells and enteroendocrine cells that reside in the top-third of the crypt [9, 58]. These cells are derived from stem cells located at the bottom of the crypt which are undifferentiated, multipotent and self-renewable and they are involved in tissue homeostasis and repair [93].

Their asymmetric division gives rise to two different daughter cells. One type is identical to the original cell, and the other has the potential to differentiate into other mature cells and they are called “progenitors”. The progenitors migrate upward the crypt to the top, proliferate and differentiate into an epithelial cell to reproduce the mature intestinal structure. These cells are always under a replacement process as they are constantly shed into the luminal space when they become senescent [69, 100]. The rate for colonic epithelium renewal is considered of 5 days and all this process is closely regulated by stem cells and always under microenvironmental influence [58, 81]. Thus, it has been calculated that over $6 \times 1{,}014$ colonocytes are produced during the individual lifetime [17, 74, 75].

Although these stem cells divide mostly asymmetrically, symmetric divisions may also occur mainly during injury, disease or neoplasia [25–27, 93].

The maintenance of the stem cell compartment, which is really important, is accurately regulated by Wnt signaling ligands, though other factors, such as the bone morphogenetic protein (BMP) antagonists gremlin 1 (GREM1) and gremlin 2 (GREM2), Notch signaling pathways, ephrin-B1 (Eph-B1) and its receptors Eph-B2 and Eph-B3, contribute to stem cell behavior, migration, and differentiation [4, 24, 51, 95].

Wnt signaling ligands are probably produced by mesenchymal cells of the myofibroblast lineage which are located in the basal lamina surrounding the crypt [32].

An intestinal crypt contains approximately 16 stem cells and harbors two distinct pools of putative stem cells. One pool is located at the crypt base and is characterized by the expression of leucine-rich repeat containing G protein-coupled receptor 5 (Lgr-5) and the other pool resides at þ4 position and consists of B lymphoma Moloney murine leukemia virus (Mo-MLV) insertion region 1 homolog (Bmi-1) and telomerase reverse transcriptase (Tert) expressing cells [54, 91].

What is still controversial is the contribution of each type of cells to the maintenance of the stem cells pool. One of the possible explanations would be the existence of a pool of equally contributing cells where each cell's behavior is defined by its environment and in terms of replacement they follow a pattern of neutral drift [54].

Other explanation could be that Lgr-5þ stem cells comprise the active population of the crypt, whereas Bmi-1þ or Tertþ cells are quiescent. The latter would represent a reserve pool with the capacity to replace Lgr-5þ cells in case of injury [91]. Or as others have proposed, Bmi-1þ cells may not have an impact on Lgr-5þ SCs, as Bmi-1 knockout mice show normal crypt morphology and have a normal intestinal epithelium [96].

What has been widely accepted is the functional relevance of the intestinal microenvironment (also called niche) in the advanced control of stem cells life cycle. This niche is formed by cellular and extracellular components aiming at ensuring the optimal conditions for stem cells survival. This is achieved by several cytokines secretion, or growth factors, and also by direct interactions [78, 93].

In fact, intestinal stem cells are also affected by components in the crypt lumen, coming from the epithelial cells or from bacterias. Moreover the subepithelial myofibroblasts are key regulators of stem cells self-renewal and differentiation, mediate the crosstalk between epithelial- mesenchyma and secrete a wide range of morphogenetic factors as Medema and Vermeulen have shown [58]. The existent interactions between epithelium and mesenchyma regulate the normal intestinal architecture and define the relevant balance between proliferation and differentiation [78, 93]. Although different pathways are involved in these interactions such as Hedgehog, BMP, Notch, and platelet-derived growth factor, Wnt/B catenin pathway is the master in controlling the relationship proliferation-differentiation in healthy and malignant intestinal epithelial cells [29, 58].

In fact Sonic Hedgehog and Wnt pathways are commonly hyper-activated in tumors and are required to sustain tumor growth. Moreover, a degree of crosstalk between these two pathways and their activation take place simultaneously [29].

6.3 Identification of Normal Intestinal Stem Cells

Intestinal stem cells are broadly defined by at least two functional properties: the capacity to perpetuate themselves by self-renewal over prolonged periods and the multipotency or the potential to generate all the differentiated cells of the intestinal origin. Chen and Leblond introduced the concept that all mature epithelial cells within the intestine derived from a single multipotent stem cell and this fact is well

documented in mouse small intestine. They identified small cycling epithelial cells interspersed between the Paneth cells, or the so-called crypt base columnar (CBC) cells, by using morphological methods in mammalian intestine [15, 16].

Later, Bjerkness and Cheng provided additional information on these specialized cells using clonal marking techniques [7].

All these researchers have hypothesized that CBC cells might represent the actual intestinal epithelial stem cells [7, 8].

Though it has been a significant recent progress in the field of stem cell biology, the identification, isolation, and characterization of colonic crypt stem cells remains elusive mainly due to the lack of specific molecular markers along with unsolved technical issues. In fact the lack of clonogenic assays and the complexity of the crypt are two relevant limitations to the retrieval of stem cells from their niche where they are interspersed among more differentiated and mature daughter cells. Subsequently, only assumptions can be made regarding their exact number and position. This is the reason why the exact identity of the intestinal stem cells has proven to be controversial over the last 30 years [104].

Several studies have been performed, mostly on small intestine, using DNA labeling techniques to use the comparatively slower cycling rate of stem cells [39]. These studies have led to the formulation of two different models regarding their position [72]. One has suggested that intestinal stem cells are located at a position +4 from the bottom of the crypts with the lowest three positions relegated to the terminally differentiated Paneth cells. Potten et al. provided evidence supporting this hypothesis by using the DNA-labeling reagents, bromo-deoxyuridine or (3H)-thymidine, on radiation-sensitive, label-retaining cells. All these studies demonstrated that the label-retaining cells were located at the +4 position in the crypt at the origin of the migratory epithelial cell column [71, 72].

Alternatively, the stem cells zone model was proposed after discovering the presence of small immature cycling cells at the crypt base among Paneth cells. These cells were named CBC and express the Wnt target gene Lgr-5 [87].

Recently, many molecules, mostly located on the cell surface, have been proposed as putative stemness markers, allowing at the same time their isolation by fluorescence-activated cell sorting [93].

The fact that stem cells in adult tissues divide at a slower rate than progenitors [22] have led to the performance of studies which have tried to identify intestinal stem cells by using indirect techniques such as long term retention of label DNA which is considered as surrogate marker of stemness. This was evidenced by different methods which allowed the identification of low mitotic index cells located at the bottom of the crypts [50, 76].

Finally, the “immortal strand hypothesis” formulated by Cairns in 1975 is also supporting the DNA label retention to identify intestinal stem cells. This hypothesis is based on the assumption that stem cells retain their original DNA strands but not the new synthesized DNA. However, this is controversial because of the demonstration that in the hematopoietic stem cell compartment, the asymmetric division of genetic material has not been yet confirmed [49].

6.4 Molecular Markers of Normal Cancer Stem Cells

Bromodeoxyuridine labeling was initially used to identify the stem cells of the colon [53]. As stated above, this was based on the assumption that stem cells divide infrequently and retain the DNA label for longer time than the more quickly dividing progenitor cells. This method of identification was replaced by the identification of specific markers, usually on the cell surface, that allow stem cells to be isolated by flow cytometry.

The RNA-binding protein Musashi-1 (Msi-1) was the first molecule identified as a putative human colon stem cell marker. In *Drosophila* this marker was found to be indispensable for asymmetric cell division of sensory organ precursor cells [61]. Something similar occurred in mice where Msi-1 was required for asymmetric distribution of intrinsic determinants in the developing of mammalian nervous system [66].

The expression of Msi-1 has also been found in mouse small intestine and in human colon crypt stem cells. In fact most Msi-1 cells were located at the bottom of the crypt of human colon, between cell positions 1 and 10 and this is a distribution that could match that of stem cells according to several reports [63, 77].

Fujimoto et al. reported that the integrin subunit 1 (CD29) was a candidate surface marker for the proliferative zone of the human colonic crypt, which includes stem cells and progenitor cells [34]. This group found that the cells located in the lower third of the crypts expressed higher levels of CD29 than others. When crypt cells were isolated by flow cytometry based on CD29 levels, 2 cell populations that had different abilities to form colonies were identified.

More recently the Wnt target gene leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) has been identified which is a unique marker of normal colon stem cells whose function is unknown but it marks actively cycling cells which contradicts the concept that stem cells are quiescent [3]. A single Lgr5 cell from the intestine could regenerate a complete crypt-like structure in vitro [82].

More recently, doublecortin and CaM kinase-like-1 (DCAMKL-1), a microtubule-associated kinase expressed in postmitotic neurons, has also been proposed as a putative colonic stem cell marker [55]. The cells expressing DCAMKL-1 are resistant to apoptosis following injury produced by ionizing radiation. In fact the descendants could divide and express at least temporarily DCAMKL-1 and only few stem cells were destroyed by apoptosis. Experiments following exposure to lethal doses of ionizing radiation showed that DCAMKL-1 does not exist in the regenerative crypt when the proliferation is at its peak, but it is recovered 7 days after injury. Moreover, by the expression of DCAMKL-1 a population of quiescent cells was identified and contrary to this, actively cycling stem cells were identified by the Lgr5 marker [55].

6.5 Cancer Stem Cells

The CSCs are defined by their capacity of endlessly self-renewal, asymmetric cell division and ability to differentiate.

The evidence of CSCs in CRC was reported in 2007 by two independent groups [65, 81] which demonstrated, by using a xenograft model of CRC cells into nude

mice, that only a small subset of tumor cells, all of them harboring the CD133 or CD44 markers, was able to originate a tumor.

In fact, when injected into an experimental animal, thousands of tumor cells are necessary generate a tumor, because only a small fraction of them, which are the CSCs, have the real ability to induce it.

The first CSC was identified in a hematologic malignancy, the human acute myeloid leukemia (AML). Bonnet and Dick [10] found that the injection of a small subset of leukaemic cells with a primitive haematopoietic progenitor phenotype harbouring CD34+ CD38− resulted in generation of leukaemias over several transplantations, contrary to the group of cells expressing the phenotype CD34+ CD38+. This fact implied self-renewal and differentiation capacities [10]. In human AML the frequency of these cells is less than 1 in 10,000. After that, CSCs have been found in some solid tumors including CRC [25, 26].

It is widely accepted that genetic instability drives malignant transformation but the recent hypothesis of CSCs as the origin of cancer considers that CSCs population may come from different sources. Normal adipose-derived stromal cells, normal stem cells or even progenitor cells or differentiated cells may give rise to CSCs. The two latter would need to acquire more genetic mutations mainly in self-renewal genes.

Anyway, it is known that normal stem cells have relatively long telomeres compared to more differentiated somatic cells, they are usually quiescent or proliferate slower than their differentiated progeny, and they have increased longevity being this the reason why they are more likely to be the targets of mutants agents which eventually would lead to the formation of CSCs [30] and it has been suggested that CSCs coming from normal stem cells are more aggressive than those from progenitor cells, though this is not definitively demonstrated yet.

Mutations in the DNA of normal adult stem cells seem to be the initial event in different types of malignant tumors. This is based on the evidence that after the isolation of these cells, these can be serially transplanted into immunodeficient mice [1].

If normal adult stem cells are the founding cells of several cancer types, then CSCs probably inherit many of their characteristics. All these CSCs are often considered chemoresistant and radioresistant which are features responsible of the failure of traditional therapy [79]. Consequently, the CSC model suggests that tumor progression, metastasis and recurrence after therapy can be driven by a rare subgroup of tumoral cells that have the capacity to self-renew, while the bulk of the tumor does not have this ability.

Therefore, the deregulation of this self-renewal process leading to stem cell expansion could be a key event in carcinogenesis because self-renewal can drive tumorigenesis and the differentiation process may contribute to tumor phenotypic heterogeneity [48, 83]. It has been clearly stated that CSCs are more tumorigenic than the bulk tumor population (which is composed by differentiated cells) and are defined mainly through the expression of specific properties, such as specific detoxification enzyme systems, molecular surface markers, and embryonic signalling pathways [1].

The self-renewal and differentiation characteristics of CSCs lead to the production of all cell types in a tumor, thereby generating wide heterogeneity [14].

Though the differentiated cells of the tumor are not usually tumorigenic due to their lack of self-renewal capacity and limited proliferation potential [36], however,

they can suffer from a switch to carcinogenesis which can occur in either the stem cells or their differentiated progeny, which sometimes will acquire the capacity of self-renew [30].

It has been proposed that in several tissues certain progenitor cells could become CSCs through a dedifferentiation process, which would occur by acquisition of stem cell properties [18].

There are further evidence supporting the role of the stem cells in carcinogenesis. A previous study showed that there are similarities seen between normal stem cells and CSCs. In addition to the features stated above such as self-renewal capacity, others include activation of anti-apoptotic genes, production of more differentiated cells, induction of angiogenesis, resistance to conventional radio- and chemotherapy (e.g., due to active telomerase expression, high ALDH expression, elevated membrane transporter activity), and ability to migrate and disseminate in metastasis [103].

Conversely, there are some important differences between these two types of cells, which also corroborate the CSC hypothesis. While normal stem cells are chromosomally stable and contain a normal diploid genome, cancer cells have a significant number of chromosomal rearrangements and are almost always characterised by aneuploidy. Moreover, cancer cells may lack cell cycle checkpoint activity that allows them to completely growth arrest. More importantly, a major difference that has been found between normal adult tissue stem cells and CSCs is that stable telomere length is maintained in malignant cells [83].

6.6 Colon Cancer Carcinogenesis

CRC is one of the best-molecularly characterized cancers due mainly to the studies performed on hereditary cases, which account for about 15 % of CRC.

In the 1990s Fearon and Vogelstein formulate the classic "adenoma-carcinoma model" [99]. In fact is widely accepted that in most cases, carcinomas arise from pre-existing adenomas. This model correlates specific genetic events with evolving tissue morphology. Every step from the normal mucosa towards the carcinoma involves specific genetic alterations which are well-known. Though this linear model has evolved to a more complex approach [35], however, the classic model still stands [2] and is characterized by the neoplastic process being initiated by APC or β-catenin mutations and tumor progression, resulting from the sequential mutation of other genes, such as *K-ras*, p53 and DCC, in the context of a growing genomic instability.

Contrary to this, the CSCs hypothesis assumes that the first mutational hit occurs in a colonic stem cell located at the bottom of the crypt that can accumulate oncogenic mutations over years and once the transformation has occurred the stem cell can divide symmetrically and asymmetrically giving rise to other CSCs and progenitors, which in turn generate other cancer cells devoid of self-renewal ability. Finally the entire niche will be colonized by mutant stem cells, and the crypt will be filled with their progeny.

This event is called "monoclonal conversion" and these proliferating cancer cells will suffer from further changes that may result in the progression of cancer. Nakamura et al. has further supported this by the observation that in patients affected by the familial adenomatous polyposis coli (FAP), which is an inherited condition with a germline mutation of the APC gene, the origin is the unicryptal or monoclonal adenoma [60].

It has also been demonstrated that two color enzyme histochemistry can be used to detect the mitochondrial-encoded cytochrome c oxidase (COX) which is frequently mutated in colonic stem cells and the nuclear DNA-encoded succinate dehydrogenase [90]. In this way a COX-deficient crypt with all lineages mutated has confirmed the ability of a single mutated stem cell to repopulate a crypt. All these studies have finally demonstrated that human colonic crypts are a clonal population coming from a multipotent stem cell.

6.7 Methods for Cancer Stem Cells Identification and Isolation in Solid Tumors

CSCs can be differentiated from the bulk part of the tumor either by their specific surface markers or by the specific pathways involved. The CD 133 antigen is commonly expressed by CSCs and the normal stem cells of the tissue of origin. Conversely, CD20 antigen which is highly express in colon tissue cells, is not expressed by the CSCs.

6.7.1 CD133

The CD133 antigen (also known in humans Prominin 1, PROML1) is a 5-transmembrane glycoprotein of 865 amino acids that is located in the membrane protrusions or microvilli in the colon. This antigen has been used as a marker to enrich for human hematopoietic stem cells [59].

Its expression has been correlated with CSC in solid tumors including retinoblastoma [57], kidney cancer [13], prostate tumor [19], colon carcinomas [65, 80].

Nonetheless, the use of CD133 to identify and isolate colon CSCs is controversial; more over it has been shown that CD133 is expressed by stem cells and more differentiated progenitor cells [85]

Though its function is unknown, it is believed to play a role in asymmetric division and self-renewal. Others have hypothesized that the localization of CD133 in the apical plasma membrane protrusions of embryonal epithelial structures [20] demonstrates that it plays a role in regulating proliferation [5] though Corbeil et al. consider it as an "organizer" of the plasma membrane topology [21].

Its tumorigenic power was evaluated by different studies. O'Brien et al. in 2007 used 17 samples of human colonic cancer (6 primary, 10 liver metastases, 1 lung metastases) to perform serial xenograft implantations in diabetic (NOD)/severe-combined immunodeficient (SCID) mice and demonstrated that only the CD133+

cells implanted for xenografts generated tumors [65]. By using immunohistochemistry (IHC) the CD133 expression varied between 1.8 and 24 % in the colon cancer samples and from 0.4 to 2.1 % in the normal colon cells. The frequency of CSC in the CD133+ cells fraction was estimated to be 1 in 262 cells.

Other projects confirmed the involvement of CD133+ cells in tumor initiation process [25, 26, 97]. The group of Luccia Ricci-Vitani et al. reported again that only the CD133+ cells generated tumors in their xenograft models. In this study, the presence of CD133+ cells was barely detectable from normal colon tissues [80]. Moreover, the level of CD133 expression correlates between the primary tumor and corresponding metastasis in 94 % of cases [42, 43].

CD133+ cells readily gave rise to tumors in mice, whereas the CD133− cell population was unable to generate tumors even after serial transplantation in mice. Moreover, tumor xenografts generated by CD133+ CSCs displayed the same morphologic features of the parental tumor and were reproducibly maintained upon serial transplantation, suggesting that the molecular heterogeneity of the original tumor was recapitulated, as demonstrated by the presence of CD133+ and CD133− cells at similar ratios to the original tumor.

It was then demonstrated that CD133 is expressed also in normal colon tissue, though at lower frequency, suggesting that CD133+ CSCs in cancer samples might result from oncogenic transformation of normal colonic stem cells.

Contrary the expression of CD133 is progressively lost during differentiation, as well as the ability to transfer the tumor into immunodeficient mice. Shmelkov et al. also questioned CD133 as a CRC-CSCs marker by using a knockin LacZ reporter mouse in which the expression of LacZ is driven by the endogenous CD133 promoter to demonstrate that CD133 expression at the mRNA level in the mouse colon is not restricted to stem cells [85]. It was then concluded that CD133 is widely expressed in human primary colon cancer, whereas the CD133− population is composed mostly of stromal and inflammatory cells.

In addition, Horst et al. have recently shown that CD133 expression correlates with poor prognosis and is an independent prognostic marker for low survival in CRC [41].

These authors demonstrated that CD133+ cells lack CK20 expression but they are positive for EpCAM which means that evaluation of CD133 and nuclear β-catenin can identify colon cancer cases with significantly reduced survival [42, 43].

Thus, the definition and identification of colon CSC remains still incomplete. As suggested by O'Brien et al., among these CD133+ cells only a few selected cells are expected to be "real CSC" [65].

The heterogeneous cell population in colon cancer is partly highlighted by its multiplicity of the genetics combinations disorders found. Hence, it is likely that among CSC, several phenotypic profiles may exist, sharing some common markers and signaling pathways.

Several studies have investigated other potential CSC markers. It is important to note, that all the presented studies focused on colon CSC identification, actually isolated a "CSC containing" subpopulation with different degree of sensitivity and specificity as there is probably no ideal single marker for CSCs in any tumor system.

6.7.2 EpCAM, CD44 and CD166

CD44 and the epithelial surface antigen EpCAM (Epithelial cell adhesion molecule) have been also used for the isolation of CSCs [25, 26]. CD44 antigen is a cell surface glycoprotein expressed on lymphocytes, monocytes and granulocytes which has also been correlated to undifferentiated cells.

The group of Dalerba et al. examined the expression profile of these two markers which previously had been described in CSCs in breast cancer [70].

EpCAM is a glycosylated 40 kDa type I transmembrane glycoprotein. Its expression in an adult's human tissue is restricted to the basolateral cell membrane of glandular, pseudo-stratified and transitional epithelia cells. Though its biological role is not fully understood, it seems to play a role as an intercellular cohesion molecule modulating cadherin-mediated adhesions and thereby adhesion strength. It is overexpressed in several cancers including CRC [92].

The study by Dalerba et al. differentiated between two main expressions' profiles, EpCAM HIGH/CD44+ and EpCAM LOW/CD44−, and measured them in colon cancer cells and normal epithelial cells. In some cancer cells the profile EpCAM HIGH/CD44+ was higher than in normal cells (mean of 1.6 % *vs* 5.4 %, respectively). Moreover, 10^4 EpCAM LOW/CD44− purified cells injected subcutaneously into NOD/SCID mice failed to form a tumor, while as few as 200–500 EpCAM HIGH/CD44+ cells were able to produce tumors.

Further subfractionation of the CD44+/EpCAM HIGH cell population by using the mesenchymal stem cell marker CD166 increased the success of the tumor xenograft. However, IHC analysis of normal colonic cells shows that CD44 expression occurs not only in the stem cell compartment at the bottom of the crypt but also in cells within the proliferative compartment, thus the specificity of CD44 for colonic stem cells remains still to be confirmed.

Recently, aldehyde dehydrogenase 1 (ALDH) has been considered a new marker for normal and malignant human colonic stem cells [45, 46]. ALDH is an enzyme involved in intracellular retinoic acid production and has been linked to cellular differentiation during development, playing a role in stem cell self-protection [23].

The enzymatic activity of ALDH was measured in the EpCAM HIGH/CD44+ and EpCAM LOW/CD44− cells and it was higher in the majority of the EpCAM HIGH/CD44+ cells. When ALDH1+ cancer cells were implanted in NOD/SCID mice generated tumor xenografts with as few as 25 cells. When using a second marker (CD44 or CD133 serially) to further select cells, the enrichment based on tumor-initiating ability was increased only moderately.

In all the studies using EpCAM HIGH/CD44+ as CSCs markers, the expression of CD133 in the selected cells was heterogeneous. But when CD133 was positive, this population included the CD44+ cells. Thus CD44+ seems to be more specific to identify CSCs while this is still a matter of debate.

When CD166 (cluster of differentiation 166) was used, it was shown that its expression was different on colon cancer cells but all tumors contained a distinct fraction of EpCAM HIGH/CD44+/CD166+ cells.

When comparing the tumorigenic potential of the fraction of CD44+/CD166+ and CD44+/CD166− cells it was found that only the CD44+/CD166+ cell population was tumorigenic [25, 26].

Others have demonstrated that using CD133 and CD44 may enhance the selection of tumor initiation cells for CRC and when injected in NOD/SCID mice, the CD44+ or CD133+ populations generated tumor, whereas the CD44− and CD133− did not [40].

The number of CD133−/CD44+ cells was too small to evaluate in a xenograft study.

To go further, others decided to discriminate the respective relevance of CD44 and CD133. Unexpectedly, it was found that with only 100 CD44+ cells a tumor was initiated in a xenograft model. Moreover, knockdown of CD44+ inhibited tumorigenicity in a xenograft model, whereas knockdown of CD133+ did not [31].

Cells that express CD133 and CD24 have clonogenic potential and multilineage differentiation and thus CD133+/CD24+ cells differentiate into goblet-like, enterocyte-like, and neuroendocrine-like cells.

6.8 CSCs Pathways and Possible Applications

CSCs are often considered chemoresistant and radioresistant which are features responsible of the failure of traditional therapy [79]. Consequently, effective anticancer drugs should target not only the tumor bulk but also specifically the CSCs. The reason why these cells are considered drug resistant is their elevated expression of the family of ATP-binding cassette (ABC) transporters and their low proliferation rate. And it is this drug resistance the reason for tumor recurrences, as these cells persist after treatment and compose the “minimal residual disease”.

To develop specific CSCs targeted therapy it would be necessary to characterize their specific signaling pathways. Though there are different implicated pathways in CSCs biology, some of them are considered major.

6.8.1 Wnt/β-Catenin

This pathway plays a critical role in cellular proliferation, differentiation, migration, apoptosis survival and apoptosis [64, 89, 94]. The Wingless-related protein (Wnt) signalling pathway is important for stem cell self-renewal, but expression of Wnt pathway inhibitors, such as axin, leads to inhibition of stem cell proliferation [64].

Moreover, Wnt proteins can help in maintaining stem cells in an undifferentiated state within their niche, and alterations in the Wnt pathway have been observed in breast and colon cancer carcinogenesis [68].

Generally the Wnt pathway is upregulated in several malignancies (around 50 %) and its regulation is done by the cytoplasmic concentration of catenin. In fact, it is a key pathway in cell development [28].

The inhibition of the Wnt/-catenin signaling pathway has been shown to be effective at blocking epidermal squamous cell carcinoma development, and a new approach to antagonise this signalling involves the stabilisation of axin and therefore maintaining the catenin destruction complex [46].

In basal-like breast cancer, the inhibition of Wnt signalling has demonstrated to block stem cell self-renewal and to repress the expression of the CDH1 repressors Slug and Twist, which in turn, block metastasis dissemination [12].

In spheroidal culture, CSCs CD133+/CD166+ showed heterogeneity in the Wnt signaling and heterogeneity in catenine location, although all these cells carried an APC mutation [98].

On microarray analysis of these CSCs CD133+/CD166+ population, two main fractions were identified: the TOP-GFP high fraction which demonstrated upregulation of the expression of stem-cell-associated genes like LGR5, and showed a higher clonogenic potential in vitro and the ability to induce tumors in immunodeficient mice. On the contrary the other population, the TOP-GFP low, expressed epithelial differentiation associated gene like mucin 2 (MUC2), cytokeratin 20 (CK20) and fatty acid binding protein 2 (FABP2) [86, 97].

In spheroidal culture each cell line remains independent and thus the regulation of the Wnt pathway is insured at least in part by the cell intrinsic characteristics.

The TOP-GFP high cells, when cultivated in a medium containing serum, they get progressive differentiation and loose CSCs markers. Moreover, if in culture with myofibroblast cell lines, their morphological and molecular differentiation was avoided and their clonogenicity was highly improved (by 50 fold). By a cytokine antibody array it was revealed that the hepatocyte growth factor (HGF) was one of the most abundant factors present in myofibroblast cell lines [86, 97].

As observed, CSCs are not only independent cells clones driving the tumor growth but their activity is highly related to their microenvironment and this relationship between the CSCs and the stromal cells is key and establishes a link between the CSCs and tumor progression. All these results have been confirmed in several solid tumors models.

The study by Zhu et al. [105] was carried out in mice model with a knock down for one or two of the CD133 alleles and showed that mice completely knockout for CD133 were viable with normal development. In the small bowel, the CD133+ expression was relatively restricted in the crypt base and overlapped with that of LGR5. When the endogenous Wnt pathway in heterozygous CD133+/CD133– mice was activated, it resulted initially in disruption of the crypt architecture and a major proliferation of CD133+ cells at the base of the crypt. The entire intestine mucosa was replaced by the progeny of these cells resulting in high-grade focal neoplasic formation.

Wnt signaling is also involved in the process of epithelial to mesenchymal transition (EMT) and invasion [58].

Therefore, Wnt pathways play an important role in cells maintenance of pluripotency, though it is also involved in differentiation of embryonic cells. This is one of the most important pathways in stem cell research and it constitutes therefore a target for new cancer therapy development.

The Hedgehog proteins carry signals between stromal and epithelial cells and they appear to be Wnt suppressors, probably through BMP, being also expressed by þ4 position stem-like cells. The truth is that its real role remains elusive [58].

6.8.2 Akt and MAPK

To assess the role of these pathways in CSCs, a cDNA GeneChip analysis has been carried out in cells CD133+ or CD133− derived from samples of metastatic CRC [102]. 321 genes were up-regulated and 65 down-regulated in CD133+ cells compared with the CD133− cells.

The gene expression confirmed that changes affecting mainly PI3K/AKT, NOTCH, MAPK and transforming growth factor (TGF)-pathways among others.

Moreover, AKT was significantly activated in CD133+ cells. A culture done in soft-agarose in the presence of the AKT inhibitor II, AKT inhibitor IV or MAPK inhibitor (U0126) showed a reduction in the ability of the CD133+ cells to form colonies by 3–11 fold [102].

There are several studies that have suggested the implication of the AKT pathway in CRC CSCs.

Toll-like receptors (TLR) are a family of transmembrane receptors that activate the MAPK pathway and colon cancer cells have demonstrated a higher expression of TLR 7, 8, 9 and 10 compared to normal colon cells. In addition, the intensity of this expression was higher for the late stage tumor. Besides, the TLR 7 and 8 were co-expressed with CD133+ in tumoral cells.

There is also an evidence of a correlation between TLR expression and tumor progression [38].

6.8.3 NOTCH

This pathway plays a relevant role in the intestinal tumor initiation in mouse models and its components are highly expressed in colon CSCs compared to the normal colonic cells. In fact this expression is critical in CSCs self-renewal ability.

Sikandar et al. performed a study in which colon CSCs treated with NOTCH inhibitors in a plate culture, could no longer generate adenocarcinoma but only disorganized cells cluster without self-renewal capacity [86].

Notch may drive tumorigenesis, because it potentiates proliferation and inhibits differentiation [93].

6.8.4 Interleukin-4

The viability of primary CRC cells after being exposed to oxaliplatin and/or 5-fluorouracil was studied by the group of Todaro et al. In vitro, cells CD133− showed a high sensitivity (dose-dependent) to these drugs whereas CD133+ were resistant, even using higher doses [92].

Though different pathways have been involved in drug resistance, the interleukin-4 (IL-4) seems to strongly modulate the chemotherapy-induced apoptosis. CRC samples showed a higher level expression of IL-4 compared to normal colon cells, mainly CD133+ cells which showed positivity for IL-4 and IL-4 receptor.

When CD133+ cells were treated with these drugs, it was observed a significant increase in overall death in vitro. The treatment with anti-IL-4 resulted in a reduction in the protein expression of anti-apoptotic molecules such as cFLIP, Bcl-xl and PED.

Moreover, when nude mice were engrafted with CD133+ cells and treated by an intraperitonal injection with IL-4DM (IL-4 Rα antagonist) followed 24 h later by chemotherapy, the co-treatment resulted in a marked synergistic effect on the tumor growth compare with single agent chemotherapy [92].

6.9 Limitations of CSCs

In a tumor where the high cell variability probably the result of complex pathways where only a subset of cells are responsible for tumor initiation and development. However, the isolation and characterization of CSCs are difficult and their driving pathways are poorly understood and therefore they need to be under further research.

The CSCs theory has been proven in xenograft experiments but studies in animal models might underestimate the frequency of cells with tumorigenic potential.

The origin of CSCs though definitively unknown, it is thought to come from tissue stem cells. In fact under specific conditions it is possible to reprogram cells to have a stem-like phenotype. Takahashi et al. showed that expression of c-Myc can convert the cells into pluripotent ones with a phenotype virtually indistinguishable from embryonic stem cells. In fact, the evidence that the proto-oncogene c-Myc could be part of the reprogramming of genes, supports the hypothesis of reprogramming a cell to have a stem-like appearance and phenotype [88].

Another limitation is related to the way to identify these CSCs. Though CD133 is one of the most efficient markers, its biological function remains still unknown and in CRC cell lines the knockdown for CD133 resulted in a significant decrease in the level of CD133 mRNA and protein expression, without any impact on rate of proliferation, migration or invasion in vitro.

Moreover, its expression is not restricted to stem cells. In fact, the analysis of CD133 knockout mice revealed that this is expressed in epithelial differentiated tissues of several adults' organs [85].

There are also tumors without CD133+ cells and in these cases some of the CD133− cells have been reported as initiating cells with contradictory phenotypic profiles such as CD133−/CD44+/CD24− [85] or CD133−/CD44−/CDX-2+/CK20+/CK7− [62].

It is then of critical relevance to be able to distinguish the best sets of markers to identify the CSCs. The CSCs may be different among divers CRC but probably sharing similar phenotypic and function specificity. Different pathways have been implicated in CSCs functions and the upregulation of them is related to tumor proliferation and self-renewal. These findings have contributed to the development

of targeted agents to downregulate these pathways and improve the tumoral chemosensitivity. The problem is that these pathways are not specifically used by CSCs and thus the efficiency and side effects of these agents is still unknown.

Conclusions

Experimental and clinical evidence support the hypothesis that in humans, the process of carcinogenesis is initiated in an adult normal stem cell. But there are still many aspects that remain to be discovered in the field of CSCs. The characterization of these cells becomes crucial for the development of more specific targeted treatments which would enhance the responsiveness to traditional therapies and eventually contribute in reducing local recurrence and metastasis and thus increase the survival.

However, the identification of these cells is still controversial and so further research needs to be done to find specific and reliable CSCs markers and a better comprehension of the molecular mechanisms underlying the biology of CSCs is mandatory.

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Chapter 7
Cancer Stem Cells in Genitourinary Cancer

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Abstract Although investigation in Cancer Stem Cells (CSC) has been more intensive in leukemia or breast cancer, genitourinary tumors, specially prostate cancer, are an important focus of attention in this field. Prostate cancer is the second leading cause of cancer death in men after lung cancer, and Cancer Stem Cells have been proposed as one of the mechanisms of resistance to hormonal treatment and chemotherapy. Epithelial to Mesenchimal Transition is believed to be associated with drug-resistance in prostate cancer, and Wnt, Notch and Hedgehog are implicated in this phenomenon.

In the case of bladder cancer it is believed that the CSCs present in urothelial tumors may originate in the basal layers of these organs. Concerning the stem cell origin of renal CSCs, the data are still discordant. The lack of CD133+ marker in renal CSCs may support the idea of an origin from a yet unidentified mesenchymal population. In this chapter authors review the most important data on the role of cancer stem cells in the initiation and development of prostate, bladder and kidney cancer.

Keywords Prostate cancer • Bladder cancer • Kidney cancer • Cancer stem cell

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7.1 Introduction

Genitourinary cancers include the kidney, bladder, and prostate. Prostate cancer is the most frequently tumor in men, and it is the second leading cause of cancer death in men after lung cancer [26, 27]. Bladder cancer is the fifth most common cancer in the world, with an incidence of 386,000 new cases per year [26, 27]. It represents the fourth most common tumor in men and the ninth in women and 150,000 deaths are caused by this tumor around the world. Renal cancer accounts for 2 % of cancers in adults. In the European Union, in 2008, 63,000 new cases were diagnosed and 26,000 people died.

The concept of Cancer Stem Cell (CSC) has been demonstrated in several human cancers including leukemia, brain tumor, breast cancer, prostate cancer, lung cancer, pancreas cancer or colon cancer. There are less data in the case of bladder cancer and renal cancer, but studies are in progress.

In many stratified polarized epithelia, such as the urothelial lining of the bladder or the prostatic epithelium, differentiation proceeds from the basement membrane toward the luminal surface. Accordingly, the basal layer is the proposed stem/progenitor compartment for urothelium responsible for generating enough cells to maintain human homeostasis. Similarly, it is believed that the CSC present in urothelial tumors may originate in the basal layers of these organs, while it has been proposed that luminal layer cells are also involved in the origin of prostate CSC.

In the following sections we review the most important data on the role of CSC in the initiation and development of prostate, bladder and kidney.

7.2 Prostate Cancer

Prostate cancer is the most frequently tumor in men, and it is the second leading cause of cancer death in men after lung cancer [27]. In patients with metastatic disease the prognosis is poor with 5 years survival below 30 %. The increase in absolute incidence can be related to the combination of an aging male population, and the widespread use of prostate-specific antigen (PSA) testing all around the world.

Hormone therapy is the mainstay in the treatment of metastatic disease. However, most patients fail to this treatment after 1 or 2 years. When the tumor progresses despite androgen deprivation it is called to be resistant to castration (CRPC) [19].

7.2.1 Origin of Prostate CSC

Human prostate is an exocrine gland with a structure composed of tubules and acini, formed by three different types of cells (basal, luminal, and neuroendocrine [NE] cells), surrounded by fibromuscular stroma. Prostatic epithelium contains three anatomically distinct epithelial cell populations that differ in their morphological characteristics, functional significance, and relevance for carcinogenesis. The

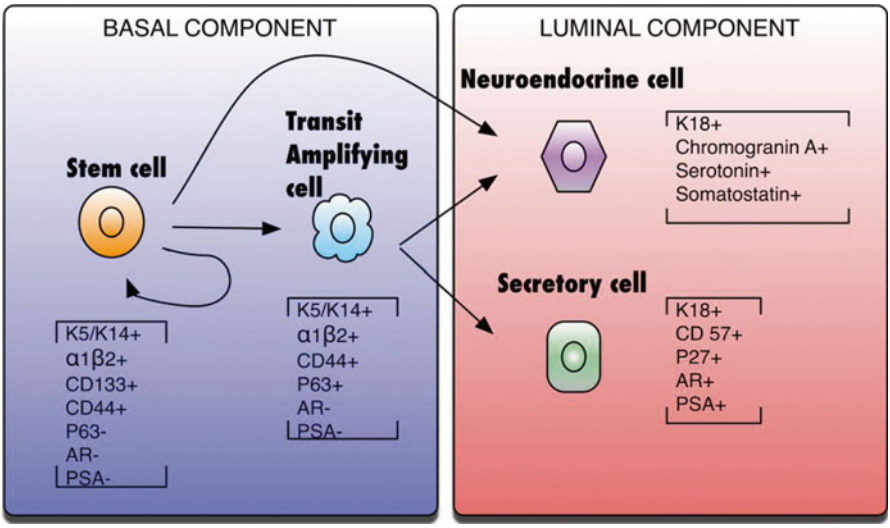

Fig. 7.1 Differentiation of prostate stem cells

differentiation patterns in prostate have been established, on the basis of keratin staining and the polarized expression of various adhesion molecules but also, critically, on the expression of proteins responsible for androgen response and cell proliferation.

Basal cells are localized beneath the luminal layer and express cytokeratina (CK) 5, CK14, CD44 and BCL-2, but express low levels of androgen receptor (AR) and no PSA or prostatic acid phosphatase (PAP) [1, 35]. Luminal cells express prostate-specific antigen PSA, PAP, AR, and cytokeratin CK 8 and 18. Neuroendocrine cells are quiescent and express specific markers like chromogranin A and do not express AR or PSA (Fig. 7.1) [48].

English et al. [17] demonstrated that the adult rodent prostate can undergo multiple rounds of castration-induced regression and testosterone-induced regeneration. Only a small population of Stem cells (SC) possesses the ability to both self-renew and differentiate while the bulk, androgen-dependent, terminally differentiated cells lack such an ability. This population is not only located in the basal layer, but also in the luminal component of the prostate. After these preliminary data on the existence of a stem cell in the prostate tissue, much of the interest of researchers focused on establishing what was the origin of these cells.

7.2.1.1 Basal Cell Origin

Initial data From Collins et al. [13] suggested that prostate tumors originate from basal cells expressing CD44+ α2β1integrin^{high} CD133+ phenotype, as this fraction exclusively had the ability to self-renew and differentiate into the mature cell types.

The p63 null mouse model suggests that epithelial development does not occur in the absence of p63, which is highly expressed in basal or progenitor layers of many epithelial tissues [3].

Single Lin− Sca-1$^+$CD133$^+$CD44$^+$CD117$^+$ cells, which are predominantly basal in the mouse and exclusively basal in human, can reconstitute prostatic ducts in renal grafts. Deletion of PTEN in PTEN-null mice is associated with an increase in p63+ basal cell numbers and the expansion of a prostate stem/progenitor-like subpopulation and consequent tumor initiation [69].

Basal cells from primary benign human prostate tissue can initiate prostate cancer in immunodeficient mice. Fusion of the TMPRSS2 to ETS the oncogenic ETS transcription factor ERG occur in 40–80 % of prostate cancers [64]. TMPRSS2–ERG is expressed in CD44 + α2β1integrinhighCD133+ cells from prostate tumors, which supports the hypothesis that the cell-of-origin of prostate cancer (PCa) is a basal stem cell. Rajasekhar et al. identified a small subset of stem-like human prostate tumor-initiating cells that do not express androgen receptor o prostate specific antigen [53]. The cells are of basal epithelial-like type and can be purified from prostate tumours based on expression of the stem cell markers TRA-1-60, CD151 and CD166. All these data are a strong line of evidence that many prostate CSC are derived from basal cells.

7.2.1.2 Luminal Cell Origin

Clinical observations that most of human prostate cancer cells express luminal cell markers support for the luminal cell of origin theory for prostate cancer initiation. Some reports have also shown that progenitor cells with luminal characteristics can initiate prostate cancer following PTEN deletion. In PTEN knockout mice, single pAkt + cells in the luminal epithelial cell layer overexpressed CK8, Sca-1, Tacstd2 and Clu; whereas basal epithelial cells were always pAkt−. Besides, Clu + Tacstd2 + Sca-1+ progenitor cells, which are candidate tumor initiating cells, were detected in the luminal epithelial cell layer of normal prostates [32].

The home box gene Nkx3-1, marks a Stem cell population that works during prostate regeneration. Functional assays of Nkx3-1 mutant mice in serial prostate regeneration suggest that Nkx3-1 is required for Stem cell maintenance. In castration-resistant Nkx3-1-expressing cells (CARNs), targeted deletion of PTEN results in rapid carcinoma formation after androgen-mediated regeneration. These results indicate that CARNs represent a luminal stem cell population that is an efficient target for oncogenic transformation in prostate cancer [70].

7.2.2 Identification of Prostate CSC

Collins et al. have identificated and isolated stem cells from human prostate epithelia based on high surface expression of integrin α2β1 and CD133 [14]. Then one possible strategy to identify prostate CSC is to use surface markers that share the same immunological profile with normal prostate stem cells.

One of these markers is CD44, an adhesion molecule with multiple functions that appears to be important in tumor dissemination and growth. CD44+ PCa cells are more proliferative, clonogenic, tumorigenic, and metastatic than the isogenic CD44− PCa cells. CD44+ PCa cells express higher mRNA levels of several 'stemness' genes including Oct-3/4, Bmi, β-catenin, and SMO. CD44+ PCa cells, which are androgen receptor (AR)−, can differentiate into AR+ tumor cells [49, 50].

CD133 is the human homolog of mouse prominin-1, a 5-trans-membrane domain glycoprotein and a cell surface protein originally found on neuroepithelial stem cells in mice. CD133 is expressed in several organs and tumors, such as hematopoietic/leukemia cells, neural/brain tumor cells and prostatic epithelium/prostate cancer. Recently, molecular profiling has been shown that integrin $\alpha 2\beta 1^{high}$/CD133+ cells exhibit an expression profile associated with embryonic stem cells. As mentioned before, Collins et al. found only tumor-derived CD133+ cells were capable of self-renewal and extensive proliferation [13]. In DU145 cells, the clones formed by CD44+ integrin$\alpha 2\beta 1^{high}$CD133+ subpopulation are remarkably different morphologically and quantitatively from those formed by integrin$\alpha 2\beta 1$−/low CD133− cells, and CD133+ cells have the capacity of self-renewal, extensive differentiation potential and high proliferative and tumorigenic potential [72].

Further, important regulators of self-renewal and differentiation such as the Wnt, Sonic hedgehog and Notch pathways were different between $\alpha 2\beta 1^{high}$/CD133− and $\alpha 2\beta 1^{high}$/CD133+ from benign prostate tissues. Despite the importance of CD133 in stem cell biology, some authors have reported that CD133 selection does not enrich for stem-like cells in PCa cell lines. Although this results may be caused by the application of different antibodies to CD133, we need to continue investigating the role of CD 133 on the development of the prostate CSC.

Rhajashekar et al. isolated TICs with stem cell-like properties from human prostate tumours [53]. These cells were androgen receptor (AR)- negative, expressed tumor rejection antigen (TRA-1-60) and exhibited active nuclear factor (NF-κB) signalling. TRA-1-60, particularly when co- expressed with CD166 and CD151, significantly enriched the prostate CSCs. CD166 and CD151 have been associated with colon epithelial CSCs and other stem- like cells in tumor stroma, respectively, and during prostate cancer progression. The triple-marker-positive (TRA-1-60+/CD151+/CD166+) subset had considerably higher capacity of in vitro sphere formation and in vivo tumor generation than the single or double positives and triple negatives, and were capable of both self-renewal and differentiation.

The specific activation of PKCα/NF-κB signalling in stem-like human prostate TICs is consistent with the previous findings that phosphorylated forms of classical PKCs (such as PKCα) and the downstream novel PKCs mediate activation of NF-κB signalling. Prostate CSC and their progenies can adapt to the persistent oxidative stress and inflammatory and hypoxic conditions prevalent in primary neoplasm by acquiring more malignant phenotypes through the activation of NF-κB and HIFs. NF-κB, HIF-1α and/or HIF-2α may induce the expression of different target gene products such as glycolytic enzymes, macrophage-inhibitory cytokine-1 (MIC-1), IL-6, cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF),

P-glycoprotein (P-gp) (also designated as multidrug resistance 1 [MDR-1 or ABCB1]), Bcl-2 and/or Bcl-xL in PC cells under normoxic and hypoxic conditions [47].

NF-κB signalling axis involving the IL-6/MCL-1 in the functional stem-like TICs is consistent with other relevant findings such as IL-6 (a NF-κB target) regulation of MCL-1 expression and mediation of cell survival responses by blocking apoptosis during late stage prostate carcinoma or activation of NF-κB via a positive feedback loop to maintain neoplastic transformation in a cell culture model system [53].

The elevation of IL-6 is consistent with studies indicating that IL-6 establish a dynamic equilibrium between CSCs and non-stem cancer cells and could convert non-stem cancer cells to CSCs in mammary and PCa models.

7.2.3 Therapeutic Targets of Prostate CSCs

Although patients with metastatic disease respond to initial suppression of gonadal androgens by medical or surgical castration, most of them eventually progress and develop a castration resistant status. This clinical situation includes patient cohorts with significantly different median survival times and different sensitivity to chemotherapy (cabazitaxel) or second hormonal manipulations (such as antiandrogen withdrawal, abiraterone acetate and corticosteroids) [12].

Current therapies have been based on the fact that most cancer cells retain similar characteristics in proliferation, invasion, and metastasis. The CSC theory allows a different approach: to cure the cancer we need to eliminate a small population of CSC. Therapeutic strategies to repair or eliminate cancer stem cells are at a preliminary stage. In determining the phenotypic differences between normal and malignant stem cells in prostate, there is a signature that could not only differentiate cancer from benign, but also stem from in transit-amplifying cells (TA). Destruction of stem and TA cells would provide a more lasting therapy than just elimination of the more differentiated progeny cells, as we currently do.

In selecting the CSC compartment within the tumor, there are three critical factors to consider:

1. Cell cultures are required to achieve a high cell number. Cultures with CSC have a short life because the genetic instability of these cells don't allow prolonged cultivation. This fact requires the use of strategies for CSC immortalization, for example using telomerase-immortalized human non-malignant prostate epithelial cells.
2. Second, we should use a different strategy to measure the decrease in the rate of cell growth, since rapid proliferation is not necessary in the stem cell compartiment. Induction of change from the AR-negative stem and TA compartments to more differentiated cells would render the stem cells also susceptible to multiple antiandrogen therapies [41].
3. Third, CSC could have inherent resistance mechanisms, to protect stem-cell integrity in tissues. Prostate CSC seem to be androgen insensitive and equipped with antiapoptotic mechanisms.

Therapeutic strategies could be directed to several critical points related to different characteristics of prostate CSC, as stated below.

Androgen receptor signaling pathways. Androgen receptor appears to play a central role in the differentiation of normal prostatic epithelium. In CRPC, AR is reactivated by a variety of mechanisms. Prostate CSC are thought to be AR-negative yet able to undergo transit amplification to generate prostatic epithelium, but then the expression of AR in CSC is still controversial. Some authors have pointed out that CD133+ PCa cells were originally reported to be AR−; however, other studies suggest that CD133+ cells responsible for tumor propagation and progression are AR+ and they are a direct target for androgen stimulation [55, 65]. New studies are required to clearly define the role of AR and androgens in prostate CSC.

AR-signaling pathways may also be a therapeutic target for prostatic stroma, which has the potential to respond to androgen in prostatic normal epithelium and in prostate cancer. Cancer cells live in a complex microenvironment that includes the extracellular matrix and cellular components. Interactions between both components have an important role for the progression of prostate cancer. Under the influence of androgen stromal cells produce several growth factors such as transforming growth factor (TGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF), that play a central role in the normal prostatic epithelium and cancer tissue. It has been reported that high levels of AR expression in epithelial cells or low levels of AR expression in stromal cells are correlated with the recurrence of prostate cancer [54].

Epithelial to Mesenchimal Transition. Epithelial to Mesenchimal Transition (EMT) is a crucial transdifferentiation process, which occurs during embryogenesis and in adult tissues following wound repair and organ remodeling in response to injure, and also occurs during cancer progression. This multistep process results in the loss of cell-to-cell adhesive properties, loss of cell polarity, and the gain of invasive and migratory mesenchymal properties [63].

EMT is believed to be associated with drug-resistance in prostate cancer [71]. During EMT, cells downregulate E-cadherin, a membrane glycoprotein involved in the adherence of adjacent cells. The loss of E-cadherin in primary tumor tissue has been linked with tumor metastasis and poor prognosis. Interactions between TGF-β and the embryonic stem-cell signaling pathways can also have an important role in maintaining the stem-cell-like characteristics of EMT-induced tumor cells.

It has been reported that the secretion of TGF-β1 and stromal cell derived factor-1 (SDF-1) by tumor stroma may cooperate for inducing the malignant transformation of non malignant cells. SDF-1 regulates the migration of putative prostate CSC, which express its receptor, CXCR4, and these effects are inhibited by anti-CXCR4 antibody in vitro [46]. Both the TGF-β and SDF-1 pathways may be essential for carcinogenesis and the progression of prostate CSC, and may be potential therapeutic targets.

Wnt, Notch and Hedgehog pathways. Wnt, Notch and Hedgehog (Hh) are all known inducers of EMT along with niche factors, such as members of the TGF-β family of cytokines.

Wnt is over-represented in CSC and plays a central role in modulating the balance between stemness and differentiation in several adult stem cell niches.

Bisson et al. found that treatment with Wnt inhibitors reduced both prostasphere size and self-renewal [4]. Whereas addition of Wnt3a increased prostasphere size and self-renewal, which was associated with a significant increase in nuclear beta-catenin, keratin 18, CD133 and CD44 expression. Therefore inhibition of Wnt signaling has the potential to reduce the self renewal of prostate cancer stem cells and improve the therapeutic outcome.

Hedgehog signaling has also been shown to promote tumor metastasis by being actively involved in the EMT. Hh exerts its effects on EMT via the upregulation of transcription factor SNAIL and downregulation of E-cadherin [29]. Binding of Hh to the transmembrane receptor Ptch1 initiates signaling via the Hh pathway. Ptch1 inhibits the receptor Smoothened (Smo) by preventing its localization to the primary cilium; in the presence of Hh, the Hh–Ptch1 complex is internalized, allowing Smo activation. Both Ciclopamyne (Hh inhibitor) as GDC-0499 (Smo inhibitor) are currently tested in phase I and II clinical trials.

MicroRNA regulation of prostate CSCs. Through an unbiased miRNA expression profiling in 5 PCSC and/or progenitor cell populations purified from prostate cancer xenografts, Liu et al. identified miR-34a, together with let-7b, to be commonly underexpressed in all marker positive cell populations [39]. Overexpression of miR-34a in bulk prostate cancer cells or purified CD44+ cells by transfecting with mature oligonucleotide mimics or infecting with lentiviral vectors encoding pre–miR-34a exerted pronounced inhibitory effects on tumor growth and metastasis in vivo. CD44 itself represented a direct and relevant down- stream target of miR-34a. Delivery of miR-34a oligos systemically through tail vein inhibited metastasis to the lung and other organs and prolonged the survival of animals bearing orthotopic human prostate cancer, indicating the therapeutic potential of this miRNA.

Puhr et al. have studied the epithelial-to-mesenchymal transition in docetaxel-resistant cells [52]. They described decreased levels of E-cadherin and upregulation of mesenchymal markers. Screening for key regulators of an epithelial phenotype revealed a significantly reduced expression of miR-200c and miR-205 in these cells. Transfection of either miRNA resulted in re-expression of E-cadherin. Tissue microarray analysis revealed a reduced E-cadherin expression in tumors after neoadjuvant chemotherapy. These data suggest that this mechanism is at least in part responsible for chemotherapy failure, with implications for the development of novel therapeutics.

7.2.4 Mechanism of Resistance

The high frequency of tumor relapse following therapy suggests the presence of residual CSC that are resistant to conventional therapy. Several mechanisms can be involved in CSC chemo and radioresistance, including expression of the ATP-binding cassette superfamily of active drug transporters [20]. This protein is related with multidrug resistance, as a result of reduced levels of drug accumulation within the CSC.

In vitro studies have shown that prostate cancer cells can recruit and activate Mesenchymal Stem Cells (MSC), and these contribute to a microenvironment that promotes osteolysis, tumor growth and inhibit docetaxel activity [5]. These effects are mediated, at least in part, by EGFR activation. The inhibition of the cross-talk between MSC and PCa cells by gefitinib, suggests a role for this drug in the treatment of prostate cancer.

Acetylation and deacetylation of histones plays important roles in the transcriptional regulation of genes in the eukaryotic cells. Histone deacetylase inhibitors (HDACIs) were found to be active in patients with hematological malignancies but results in solid tumors have been disappointing. To date, several mechanisms by which resistance are induced during the treatment of solid tumors with HDACIs have been elucidated, including increased expression of the multidrug resistance gene, MDR1, increased anti-apoptotic proteins and activating cell survival pathway.

Kong et al. have found that HDACIs Trichostatin A and Suberoylanilide hydroxamic acid could induce epithelial-to-mesenchymal transition phenotype in prostate cancer cells [31]. EMT was associated with changes in cellular morphology consistent with increased expression of transcription factors ZEB1, ZEB2 and Slug, and mesenchymal markers such as vimentin, N-cadherin and fibronectin. Moreover, Trichostatin A led to further increase in the expression of Sox2 and Nanog in PCa cells with EMT phenotype, which was associated with cancer stem-like cell characteristics consistent with increased cell motility.

Resistance to radiation therapy can be caused by increased expression of free radical scavengers by CSC. These molecules reduce intracellular levels of reactive oxygen species following radiation therapy [15]. By reducing levels of reactive oxygen species, resistant cells can evade the accumulation of cytotoxic effects of radiation therapy.

In another interesting study, Cho et al. compared the relative radiosensitivity of isolated CSC to the total population of their corresponding cell lines [11]. Irradiation of PC cell lines leads to increased numbers of CSC after a initial decrease. Prostate CSC are initially damaged by irradiation and show a recovery period of approximately 1 week and during this time properties of self-renewal and anchorage independent growth are reduced. Taken together, these data suggest that a CSC targeted therapy could improve the effect of radiotherapy.

7.3 Bladder Cancer

Bladder cancer is the fifth most common cancer in the world, with an incidence of 386,000 new cases per year. It represents the fourth most common tumor in men and the ninth in women and 150,000 deaths are caused by this tumor around the world [27]. The urothelial carcinoma represents 90 % of bladder tumors and most are tumors which do not invade muscle; however, more than half of these relapse and 10–20 % become muscle-invasive [56].

The prognosis in patients with metastatic disease is bad, with a median overall survival around 14 months [68]. Therefore, we have to know the alterations which

affect tumor development and the associated molecular pathways**,** which lead these tumors towards the epithelial-to-mesenchymal transition (EMT) and finally to the stem cell phenotype [42, 44].

7.3.1 Urothelial Stem Cells

The main role of the urothelium is to make a barrier against the exchange of substances between the blood and urine. The layer which most contributes to this function is the completely differentiated of umbrella cells. These cells, frequently multinucleated, have greatly resistant unions and underneath they have a variable number of intermediate cell layers, and a basal layer in contact with the basal lamina. Recent studies have discovered that interactions with the basal lamina are reserved to the basal layer cells and occasionally to the intermediates, but never with umbrella cells [30], which have an average lifecycle of 200 days.

Solid tumors can be treated with surgery, radiotherapy and chemotherapy. However, progression of the tumor disease occurs often and this progression may be due to the persistence of residual tumor-initiating cells, which has been described in other tumors such as breast cancer or prostate [6].

Kurzrock et al. located label-retaining cells to the basal layer of the rat bladder epithelium. Pulse labeling with bromo-deoxyuridine (BrdU) following by long-term observation allows distinguishing mitotically active transit amplifying cells from stem cells. A year after the BrdU pulse, around 10 % of the basal urothelial cells still was BrdU-positive. This strongly suggests they can be stem cells [34]. Nevertheless, a recent study, which tried to reproduce these results, did not reach its objective [75].

Up to the current date, there are no markers that distinguish urothelial stem cells from basal cells.

7.3.2 Urothelial Cancer Stem Cells

The best model to identify cancer stem cells is the use of primary tumor cells from patients, examining their capacity to form xenografts in immune-compromised mice and their capability to generate a heterogeneous population of tumor cells. This guarantees that the cells are not preselected or adapted to certain microenvironments [10].

Chan et al. found that the CD44 marker, normally expressed in urothelial basal cells, is able to identify cells which meet all the criteria of cancer stem cell (CSC) in invasive bladder cancer [9]. CD44+ cells were of small and homogenous size, with a high nuclear:cytoplasmic ratio (typical of CSC) and were able to generate the heterogeneity of the tumor of the patient. In addition, Yang et al. displayed similar findings in their study [74].

Other authors have proved other alterations related to CSC, such as the high expression of 67LR, the low expression of CD66C or the aldehyde dehydrogenase

1 A1 (ALDH1A1+) [23, 60]. This last one seems to participate actively in the maintenance of the stem cell phenotype. Su et al. used three cell lines of low-grade bladder cancer and found that ALDH1A1+ cells were highly enriched for clonogenic and tumorigenic cells. When they used CD 44 and ALDH1A1 together, there were no difference between the tumorigenicity of ALDH1A1+ CD44 – and ALDH1A1+ CD44+ cells [60].

The expression of CD 47 and cytokeratins 5 and 17 seem to play a role [6], considering CD47 a potential target for treatments.

7.3.3 Possible Implications: Prognostic and Treatment

ALDH1A1 seems to play an important prognosis role both in cancer-specific survival and globally; therefore, its high expression is associated to a considerably worse prognosis [60]. On the other hand, the expression of CD44 is also associated to a worse prognosis [9]; in an extension of this study the presence of basal bladder cancer cells (CD90+CD44+CD49f+o CK14+CK5+CK20–) is associated to a considerably worse prognosis [67].

CSC's have unique properties that allow them to survive and repopulate residual tumors after chemotherapy treatments. This makes them suitable to be studied in relation to the efficiency or resistance of different treatments. In a study in which CSC of cell lines of bladder cancer were isolated based on the expression of CD44 the findings were that, after cisplatin exposition, the CD44+ cells had a greater survival compared to CD44–, as well as a greater transformation capacity when exposed to cisplatin [61].

In another study, in this case based on the expression of ALDH1A1, it was found that cells with a high expression displayed resistance to cisplatin in vitro [18]. In addition, it has also been noted that the use of an antibody, both in vitro and in vivo, directed against one of the down regulated cytokeratins during the cell differentiation of bladder cancer, CK47, decreases the size of ganglionar and lung metastasis [73].

In conclusion, the urothelial CSCs can be originated from different original cells, and cannot be identified through a specific single marker. In the future treatments should aim towards the genetic subjacent abnormalities and aberrant molecular pathways.

7.4 Kidney Cancer

The mammalian kidney develops in three stages (pronephros, mesonephros and metanephros), and only the latter continues as the adult kidney. The kidneys arise from dorsal mesoderm, a region that originates in the pre-axial mesoderm, rich in mesenchymal cells. Mesenchymal cells are able to differentiate into epithelial cells under the influence of the ureteric bud. The induced mesenchyme in turn sends

signals to the ureteric bud to grow and divide. This mutual induction works in an ordered way to produce several generations of branches and eventually all kidney nephrons [57]. These considerations are essential to know what is the location of the stem cells that give rise to all kidney structures.

Little is known about renal stem cells. The renal stem cell search is based on the evidence that many organ systems have native stem cell populations. The search for renal stem cells is just beginning, and the unanswered questions are fundamentally whether stem cells exist in the adult kidney, where they are located and which markers can be used for characterization of renal stem cells.

Today, after demonstrating that the kidney has a dramatic ability to regenerate after injury, there are three areas of intense research on the source of renal stem cells. Although the tubular cells can regenerate themselves through the self-replication after injury, it is possible that stem cells can help to repair the damaged nephron. The cells that repopulate the tubule after injury may proceed inside the tubule (intratubular) or outside the tubule (extratubular) either from intrarenal or extrarenal sources (bone marrow-derived cells) [16, 24, 28].

7.4.1 Embryonic Kidney Stem Cells

Cellular studies show that the mesonephros is rich in stem cells; in fact, it is part of the aorto-gonad-mesonephros area, which is nowadays considered the first site of hematopoiesis in adults. The mesonephros has a close genetic and regulatory relationship with the metanephros.

In search of renal stem cells in the embryonic kidney, another potential location is the metanephric mesenchyme [16]. The metanephric mesenchyme forms nephron epithelial cells (except collecting duct epithelial, derived from the ureteric bud), myofibroblasts and smooth muscle cells. These data suggest that the mesenchyme may contain at least kidney pluripotent stem cells [2].

7.4.2 Adult Kidney Stem Cells

If the origin of stem cells is quite clear for embryonic ones, their source in adult organs is less clear and in some cases controversial. In the search for evidence of a tubular stem cell, the majority of published studies on the origin of new epithelial cells of renal tubules are based on the repair of the tubular necrosis: evidence shows that surviving epithelial cells have ability to proliferate after dedifferentiation [37, 40]. Recently, using healthy kidney, has been demonstrated the formation of new cells in the proximal tubule. These cells share characteristic features and kinetic profiles with the stem cell system [66].

It is tempting to speculate that hypoxic compartment such as the adult kidney medulla may provide a niche which allows a renal cell population to maintain their

"stemness". The medulla is the oldest region of the kidneys and, therefore, could be one of the areas in which stem cells are found.

Bone marrow has received increasing attention in recent years by scientists who hope to find a universal stem cell. There are two main reasons: bone marrow has pluripotent differentiation ability and, for organs in which a native stem-cell region has not yet been identified, such as the kidney, bone marrow can serve as an alternative source. Bone marrow has multiple types of stem cells (hematopoietic stem cells, mesenchymal stem cells, pluripotent adult progenitor cells, side population cells), and several published studies have reported differentiation when these cells are grafted onto other organs [21, 25, 36, 43, 51].

The paucity of information on the origin of the adult kidney stem cells leaves open the possibility of escaping the lineage restriction in the early embryo and later colonize specialized niches, whose function is both to maintain their potency as limit their potential lineage. The most widely accepted model, though still without foundation, for the origin of adult stem cells, assumes that they are derived after specifying its somatic lineage, which give rise to pluripotent stem cell progenitors, that colonize their respective cellular niches. The basic features of an adult stem cell are those of a clonal cell that self-renew and generate differentiated cells.

The fact that the postnatal kidney suffers a slow but constant renewal and that the adult kidney can regenerate after injury, suggests that the adult kidney contains a self-replicating cells population. So far, no cell there has been isolated in the kidney that meets the criteria of stem cell [38].

Although it is widely accepted that stem cells are tissue-specific, several studies have suggested that with respect to the kidneys may be different, and stem cells from one tissue can, under appropriate conditions, differentiate into cells of other organ, giving rise to the concept of plasticity of the stem cells. However, other researchers believe that the fusion of bone marrow-derived cells with recipient cells explains the co-localization of tracking and differentiation markers, rather than a true stem cell plasticity [33, 59, 62].

Is there a single stem cell for all types of renal cell? In simple terms, all renal cell could come from a single stem cell population. At present, the data suggest that the stroma and epithelium may share a common origin, whereas endothelial cells and their potential derivatives, smooth muscle cells, may share one second lineage.

7.4.3 Kidney Stem Cells Markers

Ongoing investigations have found two ways to identify renal stem cell markers: expression of 21 genes in metanephro, with two of these genes encoding cell surface proteins (CD24 and cadherin-11) [8], or 20 different transcripts encoding secreted molecules including known regulators of nephrogenesis (bone morphogenic protein-7 and cytokine-like factor) [45, 58]. It has been shown that renal cell tissue-derived CD133+cells are pluripotent progenitor cells, capable of self-renewal and expansion. These cells are capable of responding to local stimuli, with

differentiation to epithelial or endothelial cells, both “in vitro” and “in vivo”. Lack of expression of hematopoietic and embryonic markers, suggests that are resident stem cells, which are found in the interstitium, in the proximity of tubules [7]. Other cells with similar behavior to a renal stem cell have been isolated from rat kidney, and express vimentin, CD90, Pax-2 and Oct4, but not other markers of more differentiated cells [22].

Conclusions

Research on CSC aims to determine the interrelationship between progenitor cells and more differentiated cells. A more detailed knowledge on the phenotype and the molecular characteristics of CSC may allow to understand the mechanisms of cancer development, and help to develop new therapies in this field.It remains to identify more precisely what is the phenotype that allows us to distinguish stem cells from other tumor population, but progress in this field is indisputable in recent years.

Meanwhile we need to continue decrypting the relationship between prostate CSC and resistance mechanism to hormone therapy, chemotherapy and radiotherapy. It is also imperative to further investigate the phenomenon of epithelial mesenchymal transition, which plays an important role in tumor growth and metastasis.

For now we have less data on the role of CSCs in cancers of the bladder and kidney but certainly the contributions of many groups of researchers will allow a better understanding of the pathogenesis and treatment of genitourinary cancer.

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Chapter 8
Stem Cells in Pancreatic Cancer

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Abstract Pancreatic adenocarcinoma (PDAC), the fourth cause of cancer-related death, is highly resistant to conventional chemo and radiation therapy. Despite the efforts in therapy research and those to improve the survival of patients with pancreatic cancer in the last 10 years, there has not been major advance. The increasing study and understanding of pancreatic cancer biology has led us to discover the pancreatic cancer stem cells (CSC), a cell subpopulation with self-renewal characteristics and multipotential phenotype. They are considered the drivers of tumorigenesis and metastasis, and also responsible to confer resistance to current therapy, and repopulate the tumor after chemotherapy withdrawal. These CSCs are changing the way to understand and treat PDAC. Significant efforts have been made in CSC to define the underling mechanisms of resistance, progression and metastasis; and identify potential therapeutic targets in these cells to improve response rates and survival in patients with PDAC. The question to answer is whether this effort is working or not?

Keywords Hedhehog • Notch • Pancreatic cancer • Transforming growth factor • Wnt

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8.1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth cause of cancer-related death. In 2012, it is estimated that a total of 43,920 patients will be diagnosed with pancreatic cancer and 37,390 will die of this disease in the United States [80]. PDAC lacks of early symptoms, which leads to a late diagnosis; it also presents extensive metastasis, high resistance to chemotherapy and radiation. Despite the recent research in medical therapy and biology; the 5-year survival rate is still less than 5 % and the median survival is 4–6 months [33], only achieving with three drugs a median survival of 11 months at the cost of greater toxicity [25]. This coupled with the lack of response from the target therapy have been fostered to conduct extensive research on the pathways and mechanisms in which the PDAC progresses and metastasizes. One hypothesis suggests that current forms of chemotherapy and radiation may not target cancer stem cells. This theory has been denominated "the cancer stem cell (CSC) theory" [70]. The CSC theory attempts to explain the common clinical scenario of response during the first chemotherapy cycles followed by relapsed disease and to explain the origin of those residual cancer cells [49]. CSCs possess important properties found in their normal counterparts, most notably the ability to self-renew and undergo multilineage differentiation. Essential properties of CSC are self-renewal, evidenced as in vitro sphere formation or in vivo tumorigenicity, as well as differentiation capacity to generate the heterogeneous cancer cell population within a tumor, a process that also recapitulated in metastasis spread [3]. In addition, CSCs like their normal stem cell counterparts, are well suited to survive adverse conditions in the tissue microenvironment [8]. This tumor environment is characterized by a dense and desmoplastic stroma composed of fibrillar elements such as collagen I, activated fibroblasts, inflammatory cells, smooth muscle cells, endothelial cells, and cells of residual non-neoplastic pancreatic parenchyma [35]. It has been demonstrated in various studies that the CSC phenotype is also promoted by hypoxia, and the hypoxia-inducible factors (HIFs) provide an intriguing link between the well-established function of hypoxia in tumor growth and stem cell biology [23]. Also hypoxia enhances the self-renewal of CSCs, were the critical role for the microenvironment is the maintenance and regulation of CSCs [28]. This conjunction of stroma cells, extracellular matrix and soluble factor creating the specialized microenvironment for cell stemness is called "CSC niche". All in context makes the research of therapeutic targets for control of these CSCs niches of interest in regulating the progression of pancreatic cancer. Current criticisms to the CSC theory, came from the same model where is studied, determining that xenotransplantation have several disadvantages, the most mentioned is, that it does not provide a real microenvironment, needed for the growth and progression of pancreatic cancer. It is vital to know the difference between normal stem cells and cancer cells; the mechanisms and molecular pathways; and the microenvironment changes that lead to transformation, maintenance, progression and metastasis in CSCs in PDAC.

During tumor development, tissues accumulate a series of mutations over the years and these populations of cells would have to self-renew, clonally expand, and

acquire additional mutations. It is now widely believed that long living uncommon cells are tissue stem cells (SCs) or cells derived from them that acquire the ability to self–renew. Self-renewal, one of the defining characteristics of stem cells, is a cell division in which one or both of the resulting daughter cells remain undifferentiated, retaining the ability to give rise to another stem cell with the same capacity to proliferate as the parental cell. In addition to self-renewal, stem cells have the capacity to differentiate, generating cells in each organ [34, 84]. When mutated, they can become CSC. This type of stem cells are defined by similar characteristics, mainly their abilities to self-renew, a characteristic that drives tumorigenesis; and to differentiate in an aberrantly way, a property that generates the bulk of cells within a tumor. These self-renewing CSC might constitute only a small fraction of the cells within a tumor, with the bulk of the tumor composed of more differentiated cells that lack self-renewal capacity. CSCs may account for only a small fraction of cells (approximately 1 %) in any given tumor. The firsts solid CSCs were identified in breast tumors in 2003, and then CSCs were isolated from brain, colon, melanoma, pancreatic, prostate, ovarian, lung and gastric cancers [6].

Progress in stem cell research and the identification of potential esophageal, gastric, intestinal, colonic, hepatic and pancreatic stem cells provides hope for the use of stem cells in regenerative medicine and treatments for disease [1, 62].

8.2 Stem Cells During the Embryonic Development of the Pancreas

The pancreas is a complex gland, composed of an exocrine and endocrine tissue. The exocrine compartment is composed of acinar and ductal cells, responsible for the production and secretion of fluids and enzymes involved in digestion. The endocrine compartment contains five distinct cell types inside an islet called islets of Langerhans, which are responsible for the secretion of hormones that regulate the carbohydrate metabolism. Initially, they develop from distinct dorsal and ventral primordial that later fuse to form the mature organ. Both compartments, are believed to originate from an initial cell progenitor from the foregut endoderm, that express pancreas an duodenum homebox protein 1 (PDX1) during embryogenesis [19]. Other pathway involved is the Sonic hedgehog signaling pathway, which is inhibited in the surrounding mesenchymal tissue during the dorsal development of the pancreas, leading to the permissive expression of PDX1 [30]. Pdx1 seems to play a pivotal role in pancreatic organogenesis, contributing to all pancreatic cell lineages [78]. However, insulin and glucagon cells are present in early embryonic *Pdx1-/-* null mutant mice, suggesting that this gene is necessary but not indispensable to trigger pancreas formation; so, additional factors are needed to define the pancreas-specific characteristics. In a study by Zhou et al., using a lineage analysis, they identified multipotent pancreatic cells, localized specifically to the branching tips, recognized by a combination of markers ($Pdx1^{+}Ptf1a^{+}cMyc^{high}Cpa1^{+}$) [92]. Reichert

et al. identified a small fraction of cells with highly proliferative and multipotent capacities, which expressed Pdx1, and reside in the CD133+ pancreatic duct throughout the embryonic to the adult stages [69], further detailed to define the progeny of this cells in the adult pancreas. During the organogenesis and cell differentiation, the Pdx1 and Ptf1 expression occurs in an independent manner, influenced by the surrounding mesenchymal cells leading to the endocrine or exocrine fate. Other factor related in endocrine differentiation is Neurogenin 3 (Ngn3) [26]. Ngn3 is activated in duct-associated stem/progenitor cells that transform into alpha and/or beta-cells [75], leading to the endocrine linage, during development.

It is known that some of the stem cells appear to be in a dormant cell-cycle state in the stroma of the tissue. These tissue-specific stem cells are involved in the maintenance and/or regeneration of new tissue cells in response to physiological demands, like repair during injury. In the pancreas this regeneration involves two pathways. The first one is self-duplication of pre-existing differentiated cells; and the second one resembles some of the process that take place in the pancreas during fetal development, particularly the budding of islets adjacent to the ducts and the transient expression of PDX1 in the replicating duct cells [92]. A relationship has been found between chronic pancreatic inflammation and pancreatic cancer. This inflammatory continuous stimulus leads to the induction of tumorigenesis. Chronic pancreatitis has been identified as a risk factor for PDAC in humans. During pancreatic injury, there is controversy whether ducts might serve as the source of endocrine cells. After partial duct ligation (PDL), Pdx1 and Ngn3 become detectable in the ductal epithelium [90]. Other groups suggest that the effect of PDL or administration of streptozotocin, a beta cell-ablating agent, are not sufficient to induce expression of the complete complement of transcription factors required to induce endocrine cell neogenesis from ductal cells [42]. It is known that the cancer evolves from premalignant lesions called pancreatic intraepithelial neoplasia (PanIN). This progression from minimal dysplastic epithelium to more severe dysplasia and finally, to invasive carcinoma is paralleled by the successive accumulation of mutations [32]. The induction of tumorigenesis is through activation of Kras [24]. This Kras activation in acinar cells of the adult mouse leads to efficient mPanIN formation. In the same studies, it has been also demonstrated in vivo acinar to ductal transdifferentation in mouse models [15], implicating that those acinar cells could also represent the cell-of-origin for PDAC [83]. The emergence of PanIN lesions are associated with the appearance of an inflammatory stroma characterized by activated fibroblasts and myeloid-derived cells [14]. This leads to the conclusion that there might be a type of cell, which during injury or inflammation with a Kras activated induces proliferation and differentiation in pancreatic tissue.

8.3 Identification of Pancreatic Cancer Stem Cells

In cultured pancreatic cancer cell lines the two mainly existing technics to identify the possible cancer stem cells are cell-surface markers or Hoechst efflux. Pancreatic CSC have been identified and characterized using the surface markers, CD44,

CD24, ESA, CD133, CXCR4 and c-Met. Inside the tumor there are cell niches, where CSC subtypes that express multiples combinations of markers can co-exists. This suggestion comes from the study by Wright et al. [89], where two distinct phenotypes of stem cells in breast cancer denoted by CD44+/CD24– and CD133+ with no overlap of marker expression depending on the tumor were used to isolate the CSC populations. The two subtypes have been investigated with interesting findings. Lee et al. identified than the CSC expressing three surface markers: CD44 + CD24 + ESA+, compromised only 0.2–0.8 % of all human pancreatic cancer cells, had the highest tumorigenic potential [45]. The triple positive cells were 100-fold more tumorigenic than unsorted cells. Hermann et al., found that CD133 cells also had a potent proliferative capacity [29], comprising 1–3 % of PDAC cells analyzed in this study. The isolated CD133+ cells, formed tumors histologically indistinguishable from the original tumor in serial orthotopic xenografts in athymic mice. The obvious question was whether these markers or subgroups overlap in some cell phenotype. In the Hermann study, it is only reported an overlap of 14 % [29]. This might indicate that the CSC isolated by different research groups are not identical, and there will be required more studies to define if the combination of the four markers confers a highly enriched phenotype of CSC. Recently, it has been identified CSCs from PDAC based on ALDH activity as a more specific marker of cancer stem cells; finding that ALDH(+) and CD44+/CD24+ pancreatic CSCs are similarly tumorigenic, but ALDH(+) cells are more invasive and had worse survival [40, 68].

8.4 Molecular Signaling Pathways in Pancreatic Cancer Stem Cells

The CSC hypothesis suggests that only the cancer stem cell niche is capable of self-renewal and expansion conducting tumor as well as metastasis. The different pathways altered in this cell subset and how they contribute in the control and survival of the CSC (Fig. 8.1) have been studied. In solid organ malignancies, Notch, Wnt, PTEN, Sonic hedgehog (Hh) and BMI-1 [45] have been implicated. Many of these are also active in pancreatic cancer stem cells, and will be described in this section.

8.4.1 Notch Signaling Pathway

Notch signaling has a critical role in regulating cell-to-cell communication during embryogenesis, tissue proliferation, differentiation, and apoptosis. Activation of the Notch receptor leads to proteolytic cleavage of the intracellular domain by the enzyme gamma-secretase. The Notch intracellular domain (NICD) is translocated to the nucleus promoting the regulation of several transcription and growth factors. In pancreas it is believed that Notch plays a role in early developmental stages, delaying their differentiation until it becomes appropriate [88]. It plays a

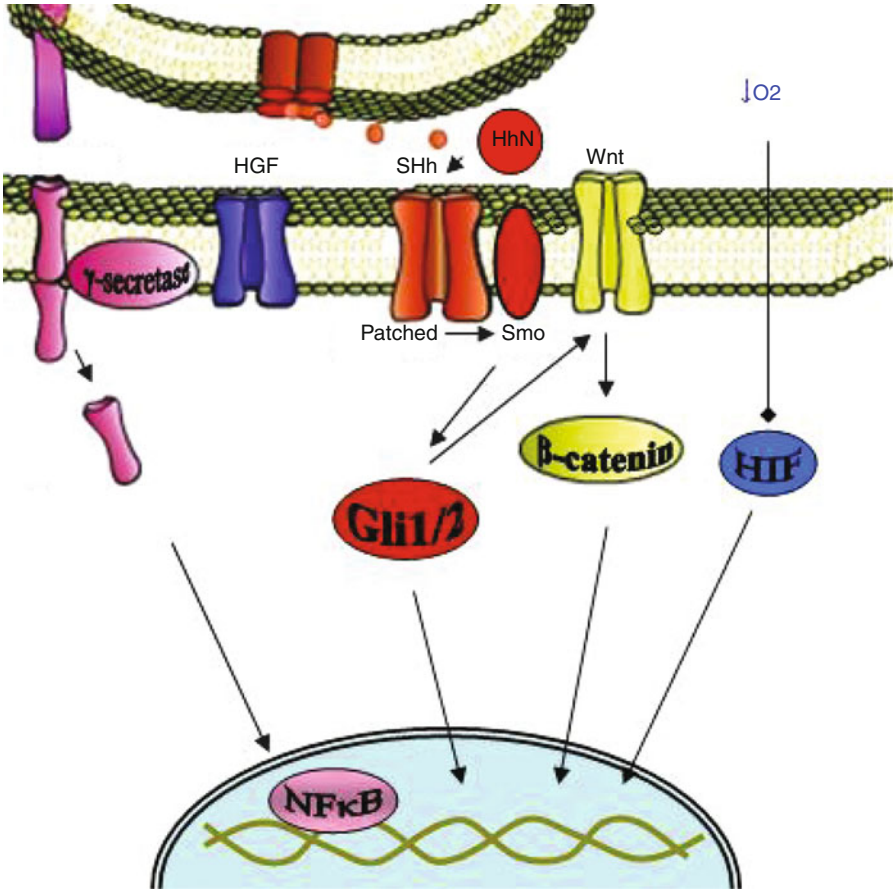

Fig. 8.1 Signaling pathways involved in CSC survival. The main cellular signaling pathways studied in pancreatic CSCs, including Wnt, Hedgehog, Wnt c-Met and HIF. All Showing their principal ligand, which during stimulation by soluble growth factors, contact with stroma cells or changes in the microenvironment leads to intracellular signaling with second messenger (in the figure the leading or immediate ones in each pathway) than translocate to the nucleus to maintain the CSCs characteristics of self-renewal, sphere formation, EMT and tumorigenesis

critical role in the development and progression of human cancer types. In pancreatic CSCs it has been reported dysregulation with high levels of Notch-1 and 2 [37]. This signaling pathway is frequently altered by up-regulated expression of Notch receptors and their ligands in many human malignancies, including pancreatic cancer, and it is suggested that they contribute to the acquisition of epithelial-mesenchymal transition (EMT) phenotype and induction of cancer stem cell phenotype [4]. It is required for PDAC initiation and the chemical inhibition of Notch activation represses PDAC development [55]. Recently, it was shown that both Notch activation and activated K-Ras signaling act cooperatively to initiate pancreatic carcinogenesis, and the progression of PanINs to invasive PDAC [15].

8.4.2 Hedgehog Signaling

Hedgehog (Hh) pathway plays a critical role in embryonic pancreatic development. Hh signaling must be suppressed to enable the pancreatic development during embryogenesis, which is initially marked by the expression of PDX1 [39], but in adult pancreas this pathway is shut down. This signaling cascade involves three Hedgehog molecules: Sonic, Indian and Desert that bind to the Patched receptor and to an uninhibited molecule, the Smoothened, a transmembrane protein that subsequently activates the Gli transcription factor and its downstream targets [17, 73]. Activation of Hh pathway has been found in various cancers including PDAC, which displays increased activity [85]. Considered to be an early and late mediator of tumorigenesis in epithelial cancers, up regulation have been demonstrated in CSCs and implicated in the progression and maintenance of pancreatic adenocarcinoma. In the mouse pancreas the overexpression of SHh leads to the development of PanIN-like lesions that possess mutant K-ras, and inhibiting Hedgehog signaling in vitro enhance apoptosis [85]. In other study, Hh signaling was blocked using cyclopamine, leading to a reduction in the percentage of cells expressing the stem cell marker ALDH, and reduction of metastasis [17]. Within the surrounding stroma, Hedgehog signaling has been shown to be important for tumor growth [73] and neovascularization [59]; therefore, targeting Hedgehog signaling can improve the delivery of gemcitabine in pancreatic cancer in vivo [62].

8.4.3 c-Met Signaling

c-Met is a receptor of the tyrosine kinase family that acts as a proto-oncogene and is stimulated by hepatocyte growth factor (HGF) to mediate motility, invasion and metastasis [56]. Also, it is well known that c-Met hyperactivation increases tumorigenicity and tumor-initiating stem cells. Some authors have reported c-Met as a stem cell marker in pancreatic tissue [29]. A recent study by Li et al., in glioblastoma CSC subpopulations revealed that CD133+ cells expressed substantially higher (up to tenfold) levels of c-Met relative to CD133− cells. The same authors concluded that c-Met signaling can dynamically regulate glioma subpopulations and expand the pool of stem-like cells [47]. In other study [60], using xenografts models, showed that c-Met^{high} cells also expresses CD44+,CD24+, CD133+ and ALDH1, but the presence of c-Met enhances tumorigenicity (when injecting the same number of cells, c-Met^{high} cells produce tumors in 35 % of the mice, while CD 133+ produces tumors in 16 % and CD44+ in 25 %) [31].

8.5 The CSC Role in Epithelial-Mesenchymal Transition and Inflammation During Pancreatic Cancer Progression

Controversy exists on the precise origin of cancer stem cells. Also still causing confusion is the use of the terms “cancer stem cells” and “cells of origin” in PDAC [41]. As reviewed in previous paragraphs, neither a ductal or centroacinar origin

can be awarded as the cell of origin of PDAC. The term cancer stem cell, does not refer to the cell of origin, but to the cell that sustains the tumor [70]. Recently, investigation has given us a clue of the relation between "the origin cell" and "the cancer stem cells" in PDAC. Theoretically, there are two subtypes of cancer stem cell pools within a tumor: intrinsic, that are thought to exist within the primary tumors from the initial stage of tumorigenesis; and the induced cancer stem cells which are differentiated cancer cells which have EMT [11]. EMT originally defined as a process of cellular reorganization essential for embryonic development [48], could be considered the transition step from the origin cell to a more "cancer stem-cell-like" phenotype. Epithelial cells are able to acquire a mesenchymal phenotype, leading to increased motility and invasion [86] during embryogenesis. In recent years it has been demonstrated also as a pathological process during the progression of various diseases, including inflammation and cancer. Increasing evidence suggest that EMT generate CSCs. In vitro studies have suggested that CSCs and EMT-type cell phenotypes overlap [20]. It has been described that Notch1 could induce EMT in pancreatic cancer cells and EMT-type cells showed increased expression of transcription factors related to mesenchymal cell markers such as vimentin, fibronectin, alpha-smooth muscle actin (SMA); and CSC surface markers CD44 and EpCAM [4]. Importantly, increased expression of fibronectin, vimentin, or N-cadherin and decreased expression of E-cadherin were correlated with invasion, metastasis, and poor survival [36]. Eighty percent of the PDAC specimens have *Snail* expression (a transcriptional factor of Notch signaling pathway) which has been involved in EMT, with others (*Slug, Zeb1/2, Twist*), they have been correlated with decreased E-cadherin levels and worse tumor grade and a poorer prognosis [66, 88, 91]. Another interaction was characterized between Hedgehog and EMT, leading to tumor progression through increased invasion [44]. In a gene expression analysis of pancreatic cells selected based on slow cycling time, a feature of stem cell populations, demonstrated increased expression of Hh and EMT associated genes [86]. Inflammation is a hallmark of cancer, considered an enabling characteristic [27], that now is well known as a initiator and promoter of EMT. There is evidence and other is being generated, trying to elucidate the role and influence of inflammation in CSC transition and the microenvironment [8]. In a study conducted by Rhim et al., it was demonstrated that inflammation induces EMT in mouse cells, suggesting that inflammation may promote cancer progression through two independent mechanisms: by facilitating changes in the microenvironment at the primary site of neoplasia and by enhancing invasion and dissemination by increasing cellular access to the circulation [71]. In recent years, the stroma or microenvironment has taken a pivotal role enhancing the desmoplastic reaction associated with pancreatic cancer. In 1998, star-shaped cells in the pancreas, called pancreatic stellate cells (PSCs), were identified and characterized [2]. In response to pancreatic injury or inflammation, these quiescent cells undergo morphologic and functional changes to become myofibroblastic-like cells [53]. PSCs have the ability to produce a wide variety of cytokines and growth factors; they produce IL-1beta, IL-6, TNF-alpha, TGF-beta1 and PDGF-BB; they also produce chemokines that contribute to the recruitment of inflammatory cells (IL-8, monocyte chemoattractant

protein [MCP]-1, and RANTES) [32]. All the evidence and recent studies support the concept that the desmoplastic response created by the cancer cells to stroma cells [35] and PSC interactions favors the progression of pancreatic cancer [53]. Recent research gives a pivotal role to all stromal cells, more related to PCS and mesenchymal stem cells, in protection from chemotherapeutic agents or preparing the distant metastasis. The PCS also appear to be responsible for the poor vascularization and angiogenesis in an hypoxia environment [52]. Importantly, the perinecrotic hypoxic microenvironment was identified as a second niche where CSC are concentrated [23, 77]. Nitric oxide secreted from endothelial cells during angiogenesis can enhanced Notch activity, leading to an increase of CSC pool and its self-renewal capacity [12]. On the other hand, inflammation can induce a direct response in CSC. Todaro et al., found in colon CSC that CD133+ cells produced and utilized IL-4 to protect themselves from apoptosis [87]. This information poses the question if the EMT-inducing factors, mostly inflammation and hypoxia, leads to CSC-like phenotype from an "origin" stem cell or promote CSC transition; and/or if they are different cells that share similar molecular characteristics and both contributes to pancreatic cancer progression.

8.6 Circulating CSC and Metastasis

8.6.1 Circulating Cancer Stem Cells

The actual clinical practice is based in the clinical manifestations, radiologic evaluations and measurement of serum tumor markers. However, this methods do not provide early enough information of metastatic spread before they become detectable [3]. Since 1869, Thomas Ashworth documented the presence of circulating tumor cells (CTC), but until recently the technology has enabled its clinical application, defining a new prognostic factor. New methods and technologies attempt to differentiate between CTC types, which can be circulating CSC and non-CSC, that as seen in previous paragraphs, both cells could be considered different cells with similar molecular characteristics. CTCs are typically present at low concentrations in cancer patients. The current common approaches for detection of CTCs in all types of cancer but used in patients with pancreatic cancer include: (1) immunological assays using antibodies directed against cell surface antigen; (2) PCR-based molecular assays for tumor-derived DNA or RNA extraction from CTCs; and (3) technologies based on physical or biological properties of cancer cells [11]. A new promising approach to isolate CTC is a technology based on microfluidic platform; call the "CTC-chip". Nagrath et al. use this chip successfully identifying CTCs in the peripheral blood of 115 of 116 patients, 15 of them with pancreatic cancer [58]. This technology provides a new and effective tool for accurate identification and measurement of CTC in a patient with cancer, and perhaps with the specific antibodies could be differentiated between CSC and CSC-like phenotypes.

In clinical practice this would allow us to differentiate which lesions in primary tumors and what CTC are prognostic for a more aggressive course of the disease.

8.6.2 Metastasis

It is becoming increasingly evident that non-stem-cell populations within tumors can transform into stem-cell [86]. There are in vivo support for the notion that EMT is associated with the initiation of a stem cell program [51] and indicate that acquisition of a CD24+/CD44+ phenotype facilitates entry into the circulation and/or survival within the bloodstream [71]. CSC may acquire a migrating phenotype through EMT in primary tumors, since the mesenchymal phenotype is usually associated with strong migration capacity while maintaining stemness, thus allowing the production of progenies during metastasis [3]. And it is hypothesized that this transformation is responsible for metastasis of solid tumors, creating a concept called "migrating stem cells" [10]. Hermann et al., showed that CD133+ characterized by the additional expression of the chemokine receptor CXCR4+ CSCs were located in the invasive front of pancreatic tumors, suggesting the higher invasive and metastatic phenotype of this cells [29]. CXCR4 is a chemokine receptor for the ligand stromal derived factor-1, a well-studied mediator of cell migration [38]. Hermann et al. also found significantly higher numbers of CD133+/CXCR4+ migrating CSC in the primary tumor of patients with lymph node metastasis (pN1+), demonstrating a close clinical correlation between this markers and the migrating capacity of CSC [29]. It has been reported that gemcitabine-sensitive PC cells, among which are L3.6pl, Colo357, BxPC-3 HPAC cells had strong epithelial markers; however, gemcitabine-resistant PC cells showed strong expression of mesenchymal markers. EMT is also responsible for the higher invasive and metastatic ability of PC cells [46]. It is now believed that the new tumor is originated from cells, which were first transformed from an epithelial to mesenchymal state and then migrated to the site of the metastasis. Another previously commented EMT inducing-factor is hypoxia. Some investigators shown the expression of CD133 increased in a glioma cell line cultured under hypoxic conditions [54]; also in gastric and colorectal cancer cells the inhibition of mTOR signaling up-regulated CD133, whereas HIF-alpha induction under hypoxic conditions down-regulated CD133 [54]. The hypoxya-induced EMT either affects CSCs only or activates or differentiated progenitors to stem-like cells or both together. In a recent study by Salnikov et al., in vitro both PDA cell lines, classified as less (CSC^{low}) and highly aggressive CSC-like cells (CSC^{high}), responded to hypoxia by altering cell morphology from an epithelial to a more fibroblastoid or mesenchymal phenotype with a higher percentage in CSC^{high}. They assume that pancreatic stem-like tumor cells may have a survival advantage under unfavorable hypoxic conditions [76]. Once that cell has moved to a new location, it can undergo mesenchymal to epithelial transition (MET), the reverse procedure [10] (Fig. 8.2).

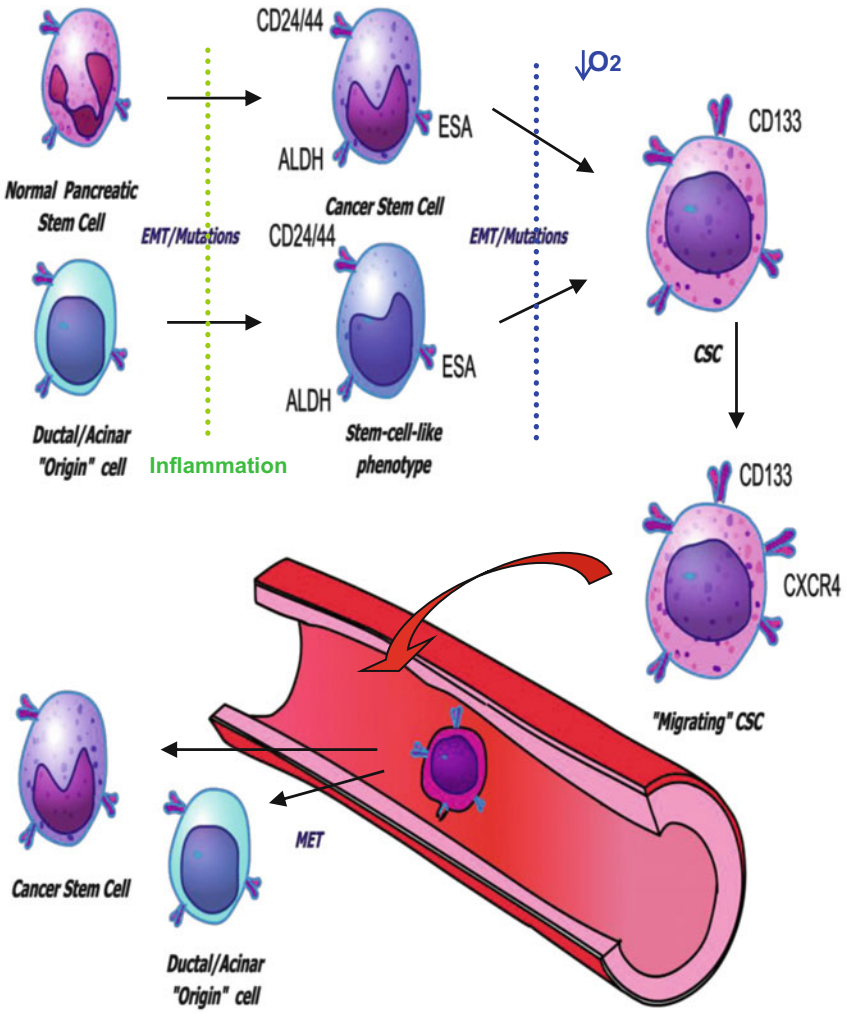

Fig. 8.2 Pancreatic CSC transitions during cancer progression. The cancer stem cells undergo a series of transformations and acquire phenotypic characteristics influenced by external factors leading to express on their membranes the clusters of differentiation that allow both the cell-cell interaction and interaction with their microenvironment. CSCs are believed to arise from both pancreatic stem cells, or from ductal/acinar cells with EMT to acquire a stem-cell-like phenotype. And finally acquire the ability to migrate through the blood stream, metastasizing to specific tissues

8.7 CSC in PDAC Chemotherapy Resistance

PDAC has inherent resistant to standard therapies like chemotherapy or radiotherapy. At present, single agent based chemotherapy (e.g. Gemcitabine) is the mainstay treatment for metastatic PDAC. Recent data indicate that in addition to Gemcitabine,

5-FU plus a platinum agent such as Oxaliplatin could be used as a therapeutic paradigm for early stage cancer patients. However, none of the available current chemotherapeutic agents have objective response rates of over 10 % [9, 67].

This made necessary to develop investigation for novel therapeutic agents. Recently, cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT)-type cells, which share molecular characteristics with CSCs, have been postulated to play critical roles in drug resistance and cancer metastasis in PDAC [9, 84]. Targeting the survival pathways in CSCs, seems to be a promising approach to improve cancer survival or even to cure it [6, 62].

8.7.1 Mechanisms of Resistance

CSCs resistance to chemo- and radiotherapy, often lead to the failure of conventional therapy and relapse [45, 64, 79]. Current chemotherapy, which prioritizes its action against differentiated cells, appears to leave some CSC intact. This may explain the relapse or distant recurrence after withdrawal of therapy. A better understanding of mechanisms that underlying CSCs resistance to treatment is necessary and may provide a more effective therapy to overcome the resistance [57].

CSC activates genetic and cellular adaptations that confers resistance to conventional therapeutic, such as: EMT, relative dormancy/slow cell cycle kinetics, efficient DNA repair, the expression of multidrug-resistance transporters, resistance to apoptosis and microenvironment that contribute to impaired drug delivery (Fig. 8.3) [5, 57, 82].

8.7.2 Relative Dormancy/Slow Cell Cycle Kinetics

During use of DNA targeting agents directed to blocks mitosis and induce cell death, the family of checkpoint kinases 1/2 (Chk1/2 kinases) surge as a potential modulator of CSCs resistance. CHk 1 and 2 are DNA damage checkpoint proteins regulating p53 and Cdc25 to mediate cell-cycle arrest/apoptosis and Cdk 1 activation respectively [61]. These kinases have higher basal and inducible activities in CSCs than non-stem cells. Additionally, the sensitivity of cells to the cytotoxic effects of radiation is cell cycle dependent, with S-phase being more resistant and the G1-S boundary and G2/M phase being more sensitive. Hedgehog pathway can control the S-phase fraction, increasing time [21].

8.7.3 Efficient DNA Repair

DNA damage cause single-strand breaks (SSB) or double-strand breaks (DSB) that limit survival and the regenerative potential of cells [7]. There are two main repair

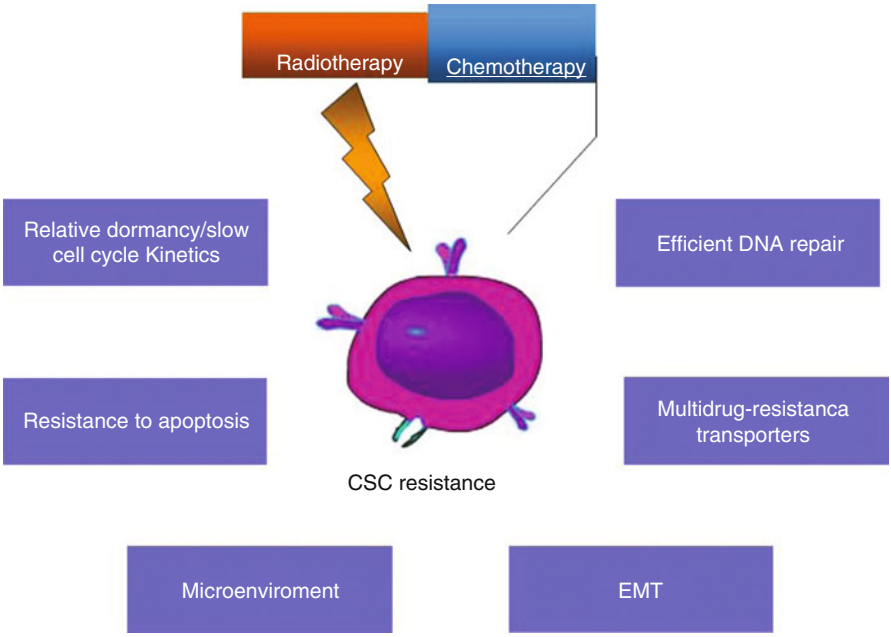

Fig. 8.3 Mechanisms leading to CSCs resistance to chemo and radiation therapy

mechanisms of DNA DSB: via non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ is an error-prone repair mechanism that enzymatically modifies the two ends of a DNA break so that they are compatible for direct ligation [22]. HR is required for a sister chromatid present in the S/G2 phase of replicating cells to provide an error-free template for DNA repair. DNA mismatch repair (MMR) and p53 deficiency are two key changes that have been associated with a resistance to chemotherapy resistance [7]. Recent studies of genomics analysis showed recurring chromosome 13 alterations via chromosomal loss or translocations involving regions containing *Ptch-1*.[21] *Ptch-1* allele showed a variety of inactivating mutations, highlighting the critical tumor suppressor function of this Hh-signaling regulator and *Ptch-1* tumor suppressor activity [13, 82].

8.7.4 The Expression of Multidrug-Resistance Transporters

CSCs may also derive resistance to chemical mutagens through the expression of drug efflux pumps for they can transport the drugs out of cells. Multidrug-resistance (*MDR*) is mediated by members of the ATP-binding cassette (*ABC*) transporter family [81]. Permeability-glycoprotein (*P-gp*) product of *mdr-1* is expressed in solid tumors and CSCs. Hh signaling regulates the expression of the *P-gp*, increasing this drug efflux transporter.

8.7.5 Resistance to Apoptosis

As previously mentioned, the Hh pathway on CSCs can opposed to apoptosis, through inferring in the intrinsic and extrinsic apoptotic cascades. Also, it has been demonstrated than pancreatic CSCs Hh signaling increased Bcl-2 expression, an antiapoptotic protein, inducing resistance to apoptosis [16].

8.8 CSCs and Therapeutics Implications

The mortality rate in PDAC has not improved since the 1970s. Gemcitabine is the currently accepted standard of care in PDAC. Efforts to improve the efficacy of gemcitabine have been mainly unsuccessful. A study by the NCI of Canada Clinical Trials Group found a survival benefit for the combination of gemcitabine and erlotinib over gemcitabine alone of 191 days compared with 177 days (hazard ratio for death 0.82; P 5 0.02) [43]. Though statistically significant, the incremental gain in overall survival was not of appreciable clinical importance. For many cancers, conventional chemotherapy is effective against the bulk, differentiated tumor cells, but after the withdrawal of chemotherapy, the residual CSCs restocked the lost cells. So it is mandatory to search novel strategies targeting theses residual CSCs responsible for disease recurrence. In pancreatic cancer, cell analyses of CD133+ CSC, showed that these cells did not undergo apoptosis, only stopped proliferating under the influence of gemcitabine, and with the drug withdrawal, immediately started to replicate and repopulated. However, the CD133 negative cells became apoptotic after the application of gemcitabine [50].

CSCs, its pathways and EMT are intriguing targets for new therapies to combat PDAC. Several clinical trials targeting CSCs, EMT and related pathways in PDAC are underway, but they remain as early as either phase I or phase II trials. They include targeting TGF-β, Hh, and two trials looking to target Notch signal (Table 8.1).

8.8.1 Targeting Hedgehog Signaling in Pancreatic Cancer

Currently, the Hh inhibitors in clinical development block the Smo target. However, other agents targeting other molecules in the CSC pathways and presenting different mechanisms of action are in research and preclinical development (Table 8.1). In pancreatic cancer only a few has been tested.

8.8.1.1 Cyclopamine

In the 1960s, this steroidal alkaloid extracted from the corn lily Veratrum californicum showed teratogenic effects, resulting in one-eyed offspring (cyclopia) in lambs. It was subsequently discovered that the active agent, cyclopamine, exerted its effects

Table 8.1 Active clinical trials targeting CSCs, EMT and related Pathways in PDAC

Pathway	Drug	Phase	Status	Disease	Comments
Hedgehog/Smo	(GDC-0449)	II	Active, not recruiting	Metastatic PDAC	Combination with gemcitabine, Nab-paclitaxel, GDC-0499 http://clinicaltrials.gov/ct2/show/NCT0108881
	(GDC-0449)	II	Recruiting	Metastatic PDAC	Combination with or without gemcitabine http://clinicaltrials.gov/ct2/show/NCT01064622
	(IPI-926)	I/II	Completed	PDAC preoperative	Combination with gemcitabine in patients with metastatic pancreatic cancer http://clinicaltrials.gov/ct2/show/NCT01130142
	(LDE-225)	I	Recruiting	Advanced PDAC	With Fluorouracil, Leucovorin, Oxaliplatin, and Irinotecan http://www.clinicaltrials.gov/ct2/show/NCT01485744
	(LDE-225)	I/II	Recruiting	Neoadjuvant PDAC	In combination With gemcitabine as neoadjuvant therapy http://www.clinicaltrials.gov/ct2/show/NCT01431794
Notch/γ-secretase	(MK0752)	I/II	Recruiting	Unresectable PDAC EC IV	Combination therapy with gemcitabine http://clinicaltrials.gov/ct2/show/NCT01098344
	(RO4929097)	II	Recruiting	Metastasic PDAC	Outcomes–survival and correlation with stem cell markers http://clinicaltrials.gov/ct2/show/NCT01232829
WNT/β-catenin	(PRI-724)	I	Recruiting	Multiple solid tumors, including PDAC	http://clinicaltrials.gov/ct2/show/NCT01302405

Information taken from: http://www.clinicaltrials.gov/ct2/home

through Hh pathway inhibition, specifically acting at the –helix bundle of Smo. In preclinical pancreatic cancer models, was founded that this drug can reduce the proportion of ALDH-expressing cells, suggesting that this subpopulation could be particularly Hh dependent [17].

8.8.1.2 Synthetic and Semi-synthetic Cyclopamine Derivatives

These derivatives have increased potency and all are oral agents. Smo inhibitors currently under investigation appear to inhibit Smo through binding at the same portion of the transmembrane segment 6 (Table 8.2)

8.8.1.3 Vismodegib ([GDC-0449] Genentech, Curis, Roche)

Results from patients with medulloblastoma and BCC on the initial Phase I study of GDC-0449 were published in the New England Journal of Medicine in 2009 [74]. Vismodegib is the first Smo- antagonist approved by the FDA in a solid tumor [49]. A current phase I study introducing vismodegib with PDAC patients, divided into two cohorts, the first one with erlotinib and the second one with gemcitabine. Both well tolerated, observing only rash in grade 4 in cohort 1 in one patient (1/15); and nausea infection and visual disturbance in cohort 2, as the main secondary effects in two patients (2/14). There were no dose-limiting toxicities, and common side effects were mild and included muscle spasms, dysgeusia, fatigue, alopecia, and nausea. Grade 3 adverse events included reversible hyponatremia, abdominal pain, fatigue, and muscle cramps [63].

8.8.1.4 IPI-926(Infinity Pharmaceuticals)

IPI-926 is the only Smo inhibitor in development, a semi-synthetic derivative of cyclopamine. The exact mechanism of action remains in investigation, but it is now well known its influence in microenvironment, by diminishing proliferation of stromal myofibroblast and increasing tumor vasculature, rather than in pancreatic tumor cells, in which it does not alter the progression of mutant KRas-induced pancreatic tumors. Also it was found in mice models that the concentration of gemcitabine metabolites was elevated by 60 % after 10 days of pretreatment with IPI-926/gemcitabine [62]. It also showed impressive metastatic blocking in orthotopic xenografts of pancreatic cancer cells [18]. Concerning human trials, an early phase clinical trial was presented at the ASCO Annual Meeting in 2011, establishing a maximum tolerated dose and tolerance. In this study, 122 untreated metastatic pancreatic cancer patients, the arm treated with gemcitabine plus placebo survived longer than gemcitabine plus IPI-926 (also known as saridegib) arm [65]. In other Phase Ib/II study of IPI-926 with gemcitabine in patients with untreated PDAC, 5 out of 16 with radiographic partial response had 5.5 months of median progression free survival.

Table 8.2 Summary of clinical findings from Phase I trials of Smoothened inhibitors in cancer

	GDC-0449 (vismodegib)	IPI-926	LDE225	BMS-833923 (XL139)	PF-0449913
Parameter	Genentech	Infinity	Novartis	BMS/exelexis	Pfizer
N (Phase I)	68	104+ (ongoing)	35+ (ongoing)	27	39+ (ongoing)
Daily doses explored (mg)	150–270–540	20–210	100–200–400–800–1500	30–60–120–240–360–540	5–10–20–40–80–120–180–270
GLI1 inhibition	Yes		Yes	Yes	Yes
Single dose t½	7 days		4 days (1–10 days)	7 days?	17–35 h

74 % of the patients were alive, 6 months after study entry [72]. The drug was well tolerated with mild effects of nausea, fatigue, muscle spasms, dysgeusia and reversible asymptomatic elevation of liver function tests.

8.8.2 Targeting Notch Signaling in Pancreatic Cancer

As seen in previous paragraphs, aberrant Notch signaling pathway is present in CSC of pancreatic cancer and contributes to tumor initiation, progression and maintenance. Their activation depends in gamma-secretase proteolysis. In a study by Plentz et al., using the gamma-secretase inhibitor, MRK0003, in different human solid tumor-derived lines showed preferential efficacy in pancreatic cell lines [66].

Conclusions

In PDAC, as in many other cancers, increasing understanding of the biology of the CSCs is leading to a new understanding in the onco and tumorigenesis. In the near future, less than 5 years, therapies targeting these cells and their environment will be included as an integral part of treatment. The new insights are also allowing us to take from preclinical to phase I/II earlier, giving some hope to PDAC patients in whom conventional therapy conventional has brought little benefit to survival. This field is still being researched and evaluated, but in pancreatic cancer appears promising

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Chapter 9
Cancer Stem Cells in Gastric Cancer

José David Gómez Rangel

Abstract Cancer Stem Cells are defined as group of cells that have the capacity to self-renewal, initiate tumor growth and metastatic potential. Gastric Cancer stem cell (GCSC) investigations are part of the expanding research field of cancer stem-cell biology of a wide variety of organs. New evidence of several years of investigation, indicate the existence of cancer stem cell. Several data suggest that a subpopulation with a defined marker show spheroid colony formation in serum-free media in vitro, as well as tumorigenic ability immunodeficient mice in vivo. There are information about possible origins of gastric cancer stem cell form an organ-specific stem cell versus a recently recognized new candidate bone marrow-derived cell (BMDC), related to malignant epithelial cells in the mouse model of Helicobacter associated gastric cancer. Bone marrow stem cells has provided enough information to explain stem cell cancer model and has open a wide field in treatment research developing a wide variety of treatment such targeted therapies and bone marrow transplantation in hematologic malignances. Using similar models, interesting evidence have been discovered in solid tumors like colon cancer, pancreatic cancer, although evidence in GCSC is inconsistent, further studies focusing on characterization and identification of GCSC biology may lead to novel strategies in diagnostics and therapeutics that could change the prognosis of gastric cancer patients.

Keywords Bone marrow-derived cell • Gastric cancer • Helicobacter • Molecular biology • Stem cell

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9.1 Introduction

Following cardiovascular diseases, cancer is considered a public health problem and at the beginning of the nineteenth century, gastric cancer was the second most common cancer around the world, with 900,000 new cases and 700,000 gastric cancer-related deaths every year. Is concerned that although the incidence and mortality rates has declined over the last years, gastric cancer still ranks as the fourth most common malignancy and the second leading cause of cancer-related death, both sexes worldwide. The late diagnosis of the disease, usually identified at the advanced stage, intrinsic resistance to chemotherapy and radiotherapy may account for the poor prognosis of Gastric cancer.

New chemotherapy regimens improves life expectancy, and despite complete resection and extensive lymphadenectomy in gastric cancer, more than 80 % of patients with advanced gastric cancer die of the disease or recurrent disease within 1 year after diagnosis.

Actually, prevention is the most effective tool for reducing the incidence and mortality of this kind of cancer. The understanding of the mechanism underlying the initiation, progression and metastatic behavior of gastric carcinoma is essential for the management of this disease.

9.2 Gastric Cancer: All of Them Are the Same Disease?

In the recent years, it has become increasingly apparent that the GC is a heterogeneous disease. The anatomical location, according to which GC can be subdivided in proximal or cardia GC and distal or non-cardia GC, identifies diseases that drastically differ in epidemiological distribution, pathologic profile, clinical presentation and prognosis.

While the incidence of distal GC shows a remarkable downward trend in incidence and mortality rate, proximal tumors have been increasing in incidence with annual rates of up to 4–5 % per year since the 1970s, mainly among males and notably in the UK, Ireland, Northern Europe, Australia and New Zealand, China, and North America. The rate at which the incidence of proximal stomach cancer has risen exceeds that of any other cancer and is taking place principally in countries with relatively low overall rates of GC. In contrast to cancer of the distal part of the stomach, proximal tumors affect the higher social classes and are associated with high BMI and obesity rather than the gastric-specific pathogen *Helicobacter pylori* (*H. pylori*) which represents the primary risk factor for distal GCs.

Helicobacter pylori infection is the major cause of gastric cancer, which remains an important health care challenge. Recent investigation in gastric stem cell or progenitor cell biology has uncovered valuable information in understanding the gastric gland renewal and maintenance of homeostasis, they also provide clues for further defining the mechanisms by which gastric cancer may originate and progress. Lgr5, Villin-promoter, TFF2-mRNA and Mist have recently been identified as gastric stem/progenitor cell markers; their identification enriched our understanding on the gastric stem cell pathobiology during chronic inflammation and metaplasia. In addition, advance in gastric cancer stem cell markers such as CD44, CD90, CD133, Musashi-1 reveal novel

Table 9.1 Differences between intestinal and diffuse gastric carcinoma

Intestinal type/differentiated	Elderly patients
	Localized in distal part of the stomach
	Frequently accompanied by liver metastases
	Adenocarcinoma is preceded by metaplastic changes
Diffuse type/undifferentiated	Poorer prognosis
	Younger patients
	Localized in cardia, but occur anywhere in the stomach
	Propensity for intra and trans-mural spread
	More poorly differentiated cells
	Adenocarcinoma is thought to arise in normal gastric mucosa

information on tumor cell behavior and disease progression implicated for therapeutics. However, two critical questions remain to be of considerable challenges for future exploration; one is how H. pylori or chronic inflammation affects gastric stem cell or their progenitors, which give rise to mucus-, acid-, pepsinogen-, and hormone-secreting cell lineages. Another one is how bacterial infection or inflammation induces oncogenic transformation and propagates into tumors. Focus on the interactions of H. pylori with gastric stem/progenitor cells and their microenvironment will be instrumental to decipher the initiation and origin of gastric cancer. Future studies in these areas will be critical to uncover molecular mechanisms of chronic inflammation-mediated oncogenic transformation and provide options for cancer prevention and intervention.

A different risk factors are known for gastric cancer, but some have been inconsistent especially vitamin C, alcohol consumption, inorganic dust, salt intake and occupational exposure to nitrosamines.

A great proportion of gastric cancer patients have adenocarcinoma (>90 %), the remaining 10 % have stromal tumor or lymphoma. According to the Lauren classification system, there are two principal types of gastric adenocarcinoma: the diffuse type (30 %) and the intestinal type (50 %), the remaining 17 % are mixed or unclassified type. Gastric adenocarcinoma can also be divided into two groups, known as "differentiated" and "undifferentiated", using the Nakamura classification system.

Principal differences between intestinal and diffuse gastric adenocarcinoma are described in Table 9.1.

Intestinal type GC, consists of gland-like structures that mimic the glandular architecture of the intestinal tract and is recognized by a series of precancerous lesions, i.e., atrophy, intestinal metaplasia, dysplasia, and finally cancer.

Intestinal-type GC typically arises in the setting of chronic gastritis. This process is known commonly as the "Correa pathway", commonly triggered by *H. pylori* infection and depends on the sustained chronic inflammation of gastric mucosa that in turn fosters a cascade of genotypic events responsible for cancer development. This traditional model of gastric carcinogenesis fits the more general theory of carcinogenesis postulated by Nowell and Vogelstein according to which the "morphological" evolution of cancer can been considered the end result of sequential accumulation of mutations in oncogenes and tumor suppressor genes in single cells. During tumor progression, transformed cells continue to acquire new mutations

with the emergence of clones that out-compete others due to increased proliferative or survival capacity and the emergence of genetically variant sub-lines with more aggressive phenotype.

Some cases of intestinal-type arise from the gastric mucosa without intestinal metaplasia (IM). Based on the type of IM, cancer phenotypes can be classified into four groups depending on the marker combinations as: complete intestinal type, incomplete intestinal type, gastric type and unclassified type. Gastric-type can be distinguished from other types because of their increased malignant potential in the incipient phase of invasion and metastasis, the mucous epithelium of the stomach represents a major barrier to the various noxious agents by means of intercellular tight junctions. Mucous epithelium and its components are also vital for physiological functions and complex communications.

The diffuse type, relatively more frequent in populations at low risk, lacks any glandular structure and usually arises in the context of a chronic inflammation but without any identifiable histological precursor lesions. While environmental factors such as diet and *H. pylori* infection strongly influence the natural history of intestinal-type GC, the role of environmental factors appears less important than the genetic influences in diffuse-type disease.

9.3 Gastric Stem Cells and Its Dark Side

The gastric mucosa and its glands show continuous bidirectional self-renewal via differentiation from stem and progenitor cells. Here, two types of gastric units, i.e., fundic and antral units, form delicate homeostatic systems. Within the last years, the central role of Sonic hedgehog (Shh) for correct self-renewal of fundic units has become much clearer. Furthermore, also the knowledge concerning the genesis of gastric cancer increased substantially. Here, chronic inflammation leads to dysregulated differentiation processes and finally to cancer. Remarkable progress has been made particularly concerning the genesis of two metaplastic cell lineages, i.e., the TFF2/spasmolytic polypeptide expressing metaplasia (SPEM) and intestinal metaplasia, which both arise in intestinal-type cancers in fundic units by dysregulated trans-differentiation of the zymogenic cell lineage. Additionally, Shh has been recognized as a target for inflammatory processes and an important player for innate immunity processes. Thus, stem cells, self-renewal, and gastric cancer are intimately linked.

In the gastric oxyntic glands, the proliferative zone encompassing the supposed gastric stem cell has been localized to the isthmus. This cells migrate bidirectionally to differentiate into gastric surface mucus cells that wrap the gastric pits, and gastric parietal and zymogenic cells that comprise the base of the gland.

The incredible ability to replace cells is vital to the maintenance of epithelial tissue. Cell regeneration from stem cells depends who have the properties of longevity, self-renewal through the generation of daughter stem cells, and differentiation in a variety of different types of mature cells. These processes of self-renewal and differentiation can occur through symmetric or asymmetric cell division.

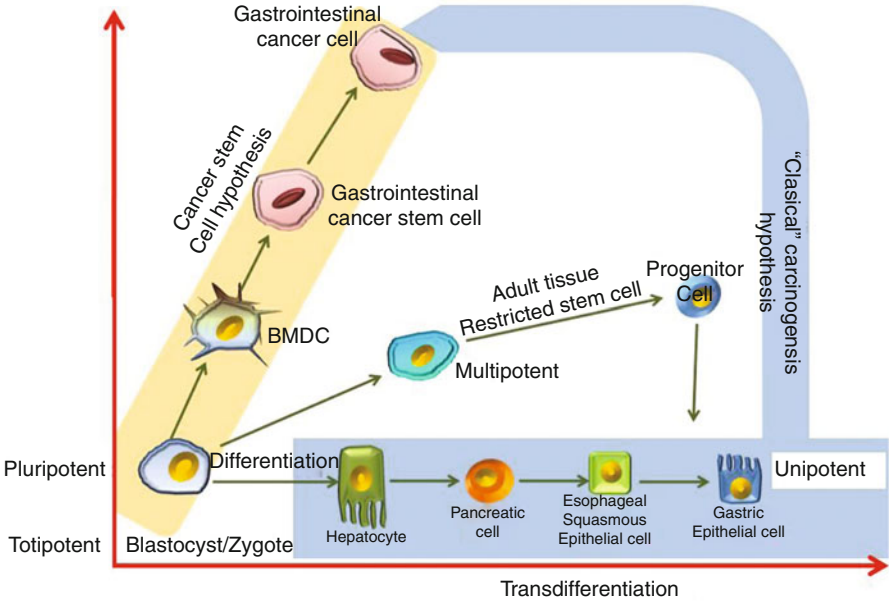

Fig. 9.1

Over the past 6 years, remarkable progress has been made in the identification and understanding of adult gastrointestinal stem cells that can be subdivided into esophageal, gastric, intestinal, colonic, hepatic and pancreatic stem cells. But there is a dark side to the presence of stem cells in the gastrointestinal tract and liver that also needs to be considered: the same self-renewal properties that allow stem cells to remain immortal and generate thousands of progeny can occasionally make their proliferation difficult to control and make them susceptible to malignant transformation.

Indeed, the cancer stem cell theory is an emerging paradigm that suggests that most cancers are sustained by aberrant stem cells that lack the normal ability to undergo terminal differentiation (Fig. 9.1). Studies published over the past 10 years have linked cancer stem cells and carcinogenesis to tissue specific stem cells, and to the accumulation of genetic alterations that occur in these tissue stem cells as they age and respond to chronic inflammation.

Normal stem cells (NSCs) are reported to exist in most tissues, including the brain, bone marrow, and probably the gastrointestinal tract. In the latter case, they are thought to possess both the self-renewal capacity and asymmetrical division capacity to generate progenitor cells which differentiate into epithelial cells. NSCs in the normal gastric mucosa are thought to be present in the proliferative zone of the neck/isthmus region, and to undergo a complex bipolar migration from the neck/isthmus region either upward or downward, becoming differentiated normal epithelial cells. NSCs in human gastric mucosa are difficult to identify due to the current lack of a useful marker. A precise definition of cancer stem cells (CSCs) is still under discussion. CSCs are generally defined as malignant cells with NSC

capacity. However, many studies of CSCs have demonstrated their rapid growth and high metastatic potential, while NSCs are thought to be slow-growing and self-renewing, and to lack functional capacities such as cell migration and attachment. Recent evidence suggests the existence of CSCs in a wide variety of solid tumors.

Under certain conditions, the local microenvironment may promote the development of gastric cancer. Thus, Helicobacter pylori infection and the accompanying chronic inflammatory processes will supply critical initiators inducing cell growth and the tissue repair response, leading to carcinogenesis. This mechanism will be discussed in light of stem cell research. Progress in stem cell research in the gastric field is still limited to experimental animal models. However, recent studies should enhance our understanding of human cancer biology, and provide novel tools for the treatment of incurable gastric cancer.

9.4 Gastric Cancer Stem Cells: Facts and Reality

Gastric cancer stem cells (CSCs) hypothesizes that cancer derives from a stem cell compartment that undergoes an abnormal and deficient process of organogenesis. Most of them share important properties with normal tissue stem cells including self-renewal and differentiation into the heterogeneous non-tumorigenic cancer cell types that constitute the bulk of the tumor. In fact those cells are relatively refractory to therapies that have been developed to eradicate the rapidly dividing cells that constitute the majority of the non-stem cell component of tumors, thus explaining why anti-cancer therapies are far from curative and why relapses of cancer are common.

The existence of CSCs had been hypothesized for many decades. However, it was not until 1994 that malignant stem cells were isolated from patients with acute myeloid leukemia, in which a rare subset comprising 0.01–1 % of the total population induced leukemia when transplanted into immunodeficient mice. Since this first experimental evidence, growing attention has been paid to the identification of a possible CSC in solid tumors.

To date, two methods are universally recognized to identify CSCs. One is an in vitro method termed “spheroid colony formation,” and the other is an in vivo method that involves the implantation of candidate CSCs under the skin or within specific organ sites (e.g., orthotopic) of immunodeficient mice (e.g., NOD/SCID mice, nude mice, Rag2/-C double-mutant mice).

The growth of spherical colonies after a few weeks is considered indicative of self-renewal ability and would be consistent with a CSC phenotype although the growth of cells in immunodeficient mice is needed to demonstrate true tumourigenicity and is generally considered the gold standard for proving the existence of CSCs.

9.5 Actual Evidence in Gastric Cancer Stem Cell

Cancer Stem Cells hypothesis, appeared more than a century ago when a number of European pathologists observed that tumors were composed of a heterogeneous mixture of partially differentiated cell types, similar in many respects to a normal organ. John E. Dick et al. first demonstrated the existence of CSCs more than a decade ago, when they proved the hypothesis to be largely true for human acute myeloid leukemia. The leukemic stem cell, which was defined as specific markers of CD34+/CD38−, could serially reproduce the disease in immunodeficient mice, and it was consistent with their properties of self-renewal and longevity.

Despite some limitations, the growth of tumor cells with defined markers in immunodeficient mice has become the gold standard for identifying a CSC in other solid tumors such as breast cancer, head and neck cancer brain cancer, melanoma, prostate cancer, pancreatic cancer, colon cancer and others. An American Association for Cancer Research workshop, a working group used the available data to create a consensus definition of the CSC as "cells within a tumor that possess the capacity for self-renewal and that can cause the heterogeneous lineages of cancer cells that constitute the tumor."

First evidence of the existence of a CSC component in GC came from a study by Takaishi and colleagues who analyzed a panel of human GC cell lines (i.e., AGS, NCI-N87, MKN-28, MKN-45 and MKN-74) and identified putative cancer initiating cells within a CD44+ cellular fraction. Consistent with the standard definition of CSCs, CD44+ cells isolated from MKN-45, MKN-74 and N-87 GC cell lines formed spheroid colonies under non adherent conditions in serum-free media and xenograft tumors in the stomach and skin of SCID mice.

At the same time, Fukuda et al. described putative gastric tumor-initiating cells by isolating and characterizing the so-called side population (SP) in five human GC cell lines (MKN45, KATOIII, MKN74, MKN28 and MKN1) and three cases of primary human GCs. SP cells, firstly described by Goodell, are a small subpopulation of cells with enriched stem cell activity and a distinctive expression profile of the ATP-binding cassette (ABC) transporter.

Due to the presence of ABC transporters, SP cells are characteristically refractory to Hoechst 33342 dye-staining and resistant to certain drugs. They have been isolated from numerous human solid cancers such as lung cancer, mesenchimal neoplasms, acute myelogenous leukemia, neuroblastoma and glioma.

Two sets of markers have emerged as the most useful for the identification of cancer stem cells in a variety of systems: CD44 and prominin-1. CD44 is a class I transmembrane glycoprotein that can act as a receptor for extracellular matrices such as hyaluronic acid, and is a known downstream target of the Wnt/β-catenin pathway. It was the first marker identified for a solid tumor stem cell found in a study of tumorigenic breast cancer. These cancer stem cells expressed CD44, but not CD24, another adhesion molecule, and classical lineage markers. CD44+ cells

exhibited the stem cell properties of selfrenewal and the ability to form differentiated progeny of CD44- cells. In addition, CD44 knockdown reduced the efficiency of spheroid colony formation as well as the size of xenograft tumors. Finally, in a mouse model of *Helicobacter*-dependent gastric carcinogenesis, INS-GAS mice infected with *Helicobacter felis* developed invasive gastric lesions strongly positive for CD44 immunostaining, especially at the invading edge of the tumors.

The role of CD133 as a marker of CSCs has been documented in several human neoplasms; its expression seems to predict unfavourable prognosis. Novel therapeutic strategies aimed at targeting molecular pathways critical for CD133+ CSCs survival are being examined.

9.6 Importance of Tumor Microenvironment

The natural history of gastric cancer (GC) differs according to anatomic site of origin and histological type. For example, intestinal type GC represents the end result of a multistep process triggered by *H. pylori* infection and evolving in the context of a chronic inflammatory state of the gastric mucosa. Therefore, the absence of an appropriate microenvironment could explain the inability of CSCs to reproduce the structural complexity of the primary gastric tumors when injected under the skin or orthotopically in immunocompromised animal models. It is noteworthy that a sustained chronic inflammation plays an active and primary role in transforming tissue stem cells into tumor cells and GC represents the typical tumor system where synergy between *H. pylori* infection, inflammation and host factors is required for effective carcinogenesis. In addition, murine studies have demonstrated that the induction of preneoplasia correlates better with the type of inflammatory response than with the strain of *H. pylori*, again emphasizing the functional importance of the inflammation compared to bacterial factors.

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that participate in the structural and functional maintenance of connective tissues under normal homeostasis. They also act as trophic mediators during tissue repair, generating bioactive molecules that help in tissue regeneration following injury. MSCs serve comparable roles in cases of malignancy and are becoming increasingly appreciated as critical components of the tumor microenvironment. MSCs home to developing tumors with great affinity, where they exacerbate cancer cell proliferation, motility, invasion and metastasis, foster angiogenesis, promote tumor desmoplasia and suppress anti-tumor immune responses. These multifaceted roles emerge as a product of reciprocal interactions occurring between MSCs and cancer cells and serve to alter the tumor milieu, setting into motion a dynamic co-evolution of both tumor and stromal tissues that favors tumor progression. Here, we summarize our current knowledge about the involvement of MSCs in cancer pathogenesis and review accumulating evidence that have placed them at the center of the pro-malignant tumor stroma.

Thus, further work is needed to define the best markers and model systems used for studies of cancer stem cell populations. Nevertheless, it is thought that specifically targeting cancer stem cells may allow more effective therapy for cancer, a notion supported by studies published in 2009 with pancreatic cancer stem cells, when a combination of blocking both sonic hedgehog and mTOR (mammalian target of rapamycin) signaling and standard chemotherapy seemed to eliminate pancreatic cancer stem cells.

9.7 Participation of Bone Marrow Derived Cells (BMDCS)

Tissue-restricted adult stem cells have for many years been the obvious candidate as a source of cancer stem cells, since in a chronically inflamed environment it is thought that they may slowly acquire a series of genetic and epigenetic changes that lead to loss of growth control and apoptotic programs, and finally the emergence of cancer stem cells. However, recently BMDCs have been proposed as an alternative candidate for precursors of cancer stem cells. BMDCs, although not pluripotent like ESCs have a somewhat wider range of plasticity than many tissue-restricted stem cells and tend to migrate to peripheral organs as a result of inflammation and tissue injury. The differentiation pattern and growth regulation of these migrating BMDCs may depend largely on local environmental signals.

The identification of circulating progenitor cells capable of functioning as lineage-specific stem cells (such as endothelial progenitors) has raised questions as to whether distinct and unique stem cell populations exist for each organ or tissue, or whether a more centralized source of stem cells exists, with the organ-specific niche being the ultimate determinant of stem cell function.

Chronic inflammatory stress and injury can lead to the recruitment of circulating progenitors to the gastric epithelium where they may engraft and contribute to the tumor mass. Bone marrow-derived epithelial cells have been identified in the lung, gastrointestinal tract and skin of mice after transplantation of a single purified hematopoietic BMDC.

This model might be restricted to cancers that arise after destruction of inflammatory tissue and it remains unclear how BMDCs undergo malignant conversion after recruitment to the gastric mucosa. Several studies have suggested that the contribution of BMDCs to the epithelium, and possibly to tumorigenesis, may be explained by fusion between a BMDC and a peripheral tissue cell. Moreover, a number of reports have shown that BMDCs can contribute to epithelial cancers.

Furthermore, bone marrow-derived endothelial progenitor cells can contribute directly to angiogenesis in tumor formation. Malignant transformation and the continued growth of a malignant cell requires a fertile microenvironment. Myofibroblasts and endothelial cells have been shown to derive, in part, from circulating BMDCs. Inflammatory cells and carcinomaassociated fibroblasts are important cells within the peritumoral stroma, and help to promote an environment permissive for tumor

growth, invasion and angiogenesis. Together with the tumor cells, they release factors responsible for the mobilization of bone marrow-derived endothelial progenitor cells and induce them to migrate and become incorporated into the developing vasculature of the tumor.

9.8 Conclusion

Gastric cancer represents a world health problem, efforts of identify an alternative origin as carcinogenesis mechanism like gastric cancer stem cells have as porpoise to develop targeted therapies to improve results in an mortal cancer like this. At the moment, results of different research groups are inconsistent to demonstrate specific markers for the identification of gastric cancer stem cells.

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Chapter 10
Cancer Stem Cells in Melanoma

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Abstract The Cancer Stem Cells (CSC) are a subpopulation of tumoral cells characterized by the ability to self-renew and to establish tumours upon transplantation, to remain quiescent for long time and to have an innate resistance to chemotherapy and radiotherapy. These features suggest that they are responsible for relapse and metastasis. In melanoma, subsets of tumoral cells with these characteristics have been identified using CSC markers such as ALDH1, CD133, ABCB5. Wnt, Notch and Hegdohog are signaling pathways involved in the biology of CSC, which are highly conserved through evolution. Although the available evidence is limited, it seems to be equally important for melanoma stem cells. Many studies have associated high levels of CSC biomarkers expression with adverse prognosis of melanoma. Knowledge of the CSC biomarkers and its signaling pathways has opened research pathway for the development of new therapies targeted to CSC. The anti-CD20 antibody, Rituximab, and immunotherapy with dendritic cells immunized against antigens of the CSC, have documented the first positive results of efficacy in melanoma. The current evidence on their CSC biomarkers, major molecular pathways involved in its biology, prognostic value and potential utility as a therapeutic target will be reviewed in this chapter.

Keywords Biomarkers • Cancer stem cells • CSC • Hegdohog • Melanoma • Notch • Wnt

10.1 Introduction

Melanoma is the most aggressive form of skin cancer, and its incidence is increasing worldwide. About 160,000 new cases of melanoma are diagnosed and 48,000 melanoma related deaths occur worldwide each year [1, 2]. Among cancers in patients

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under 40 years of age, the incidence of melanoma is the most common, after breast cancer for women and leukemia for men.

Melanoma is highly resistant to conventional radio and chemotherapies. Before 2010, no systemic therapy to improve overall survival among patients with metastatic melanoma had been shown, and the median overall survival was from 6 to 10 months [3]. In 2010, Ipilimumab, a monoclonal antibody which blocks cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), demonstrated an improvement in overall survival to 10.1 months, with a response rate of 10.8 % [4, 5].

On the other hand, in 2002, investigators at the Sanger Institute discovered that mutations in the gene encoding the serin-threonine protein kinase B-RAF occurred in 50–60 % of melanomas. Melanomas carrying a BRAF V600E mutation constitutively activate the mitogen-activated protein kinase (MAPK) pathway, promoting cell proliferation and preventing apoptosis. In recent years, small molecules which block V600E-BRAF mutation have been developed. The BRAF inhibitors (Vemurafenib, Dabrafenib) have been associated with improved rates of progression-free survival (6.9 vs. 1.6 months; HR 0.38, $p<0.001$) and overall survival (13.6 vs. 9.7 months; HR 0.7, $p<0.001$), with higher overall response rates (57 % in Vemurafenib Phase III Trial) in patients with BRAF-mutated melanoma [6, 7].

However, despite these advances, it did not success in curing metastatic disease and treatments remain palliative. The existence of a minor subpopulation of cells, called Cancer Stem Cells (CSC), responsible for the tumour initiation and progression, which has the capacity to stay quiescent for a long time and to be more resistant to chemotherapy and radiation, could be the cause of the treatment failure.

10.2 The Cancer Stem Cell Theory

A decade ago, The Cancer Stem Cell theory proposed that tumours are heterogenic and contain a subset of cells with the capacity of self-renew and generate a differentiated progeny, the Cancer Stem Cells. These cells have also an innate resistance to cytotoxic agents. They were first identified in haematopoietic malignancies and secondly in a broad spectrum of solid tumors, including those of the breast, colon and brain [8, 9]. In melanoma CSC were first identified in 2007, using different putative CSC markers, as CD20, CD133, ABCG2 and ABCB5 [10, 11].

CSC are defined by two minimal properties; the first of them is the capability to re-grow the tumour which they were isolated from; the second property is the multipotency of lineage differentiation. Operationally, CSC must be able to grow continuously even after multiple passages in vitro and to form tumours when transplanted at low-multiplicities into incompetent recipient mice which fully recapitulate the original heterogeneity of cell types observed in the primary lesions they are derived from [9].

The CSC behavior is regulated by extrinsic factors. The CSC niche may influence the self-renewal rate, the symmetric vs. asymmetric cell division, the cell proliferation, migration and differentiation. Over the last few years, it has been demonstrated

that most of cells in the tumor tissue have the potential to adopt a stem cell-like phenotype, regardless of whether cells exhibit a more differentiated or proliferative phenotype at one stage, regulated by the environment. This phenomenon is called phenotype-switching [12, 13].

The epithelial-to-mesenchymal transition (EMT) is the capacity of epithelial cells to acquire mesenchymal traits under the influence of specific environmental cues, to underlie local invasion into surrounding tissues and systemic dissemination to distant organ sites. In cancer, EMT is a crucial step towards invasiveness and metastasis, and is strongly associated with poor clinical outcome. Recent studies have shown that EMT can induce differentiated cancer cells into CSC-like state [14]. This observation also suggested that CSCs may underlie local and distant metastases by acquiring mesenchymal features which would greatly facilitate systemic dissemination from the primary mass. In fact, many investigators have established a correlation between the presence of CSCs in primary tumour and increased metastasis incidence [15–17].

Along this chapter, we will review the most important CSC biomarkers which are studied in melanoma, the major molecular pathways involved in the biology of melanoma stem cells, their prognostic significance and their therapeutic implications.

10.3 Biomarkers in Melanoma Stem Cells

The analysis of different CSC markers, previously known in other tumours, has been used in melanoma to identify subpopulations of cells with features of cancer stem cell in recent years [18–21]. However, two recent studies which used a severely immunocompromised mouse model suggested a high frequency of tumour-initiating cells in melanoma. Nevertheless, these studies were not successful in finding any correlation between a specific phenotype and a tumour-initiating ability and led to question the existence of melanoma stem cells [22, 23]. It has been proposed that the enzymatic digestion used for tumour cell preparation, the location of transplantation or the influence of the immune system could explain the discrepancy between the studies related to this issue [24].

The current and most relevant biomarkers will be reviewed along this essay.

10.3.1 Prominin-1 (CD 133)

CD133 also known as prominin 1, is a transmembrane glycoprotein normally expressed on undifferentiated cells including stem cells, recently used to isolate and define cancer stem cells from various solid tumours. Despite its unknown function in stem cell biology, it is one of the most studied biomarkers in melanoma as well.

Monzani et al. analysed the expression of CD133 in seven human melanoma specimens and less than 1 % of cells positive for CD133 was found. Afterwards,

to test the tumorigenic capacity of the CD133+ cells CD133+ and CD133− cells were injected in NOD/SCID mice. Only the mice which were injected with CD133+ cells developed a detectable tumour, whereas the CD133− fraction failed to regenerate tumours, a fact which suggested that the tumorigenic potential of melanoma seems to be retained only in cells expressing CD133 [15]. Similarly, Frank et al. shown that only 0.5–2 % of melanoma cells co-expresses CD133 and ABCB5 and exhibits stem cells properties in vivo, demonstrating that a distinct subset of melanocytic cells are marked by CD133 progenitor phenotype [19].

Klein et al. evaluated the expression of CD133, CD166 and nestin in 226 melanocytic lesions including melanocytic nevi, in situ, invasive and metastatic melanomas. Within this study significant increases in the expression of these markers during the melanoma progression compared with banal nevi were observed and an increase of melanoma aggressiveness when the expression of these markers were higher was also reported [18].

The Vasculogenic mimicry (VM) represents the formation of perfusion pathways by tumour cells and their presence in tumours is associated with adverse outcome [25]. Recently, it has been reported that CD133 and ABCB5 subpopulations are colocalized in melanomas in perivascular niches which contain CD144 (VE-cadherin) forming VM, indicating that CD133 cells act as stem-like cells driving tumour growth by promoting vasculogenic mimicry phenomenon [26].

10.3.2 ABCB5

ABCB5 belongs to the superfamily of active transmembrane transporters which act as ATP-dependant pumps in order to transport a variety of endogenous and exogenous compounds out of the cell. ABCB5 was first described by Frank et al. as a regulator of cell fusion in normal skin progenitor cells and the most important mediator of chemoresistance to doxorubicin in malignant melanoma cell lines demonstrating that ABCB5+ cells exhibit lower drug accumulation than ABCB5− [19, 27].

In 2010, it was observed that circulating melanoma cells isolated from the peripheral circulation of melanoma patients expressing ABCB5 were tumorigenic and able to form metastases in animal model in vivo. Using a xenotransplantation model in mice, Schatton et al. also reported that the majority of ABCB5 positive subpopulation cells were able to form tumours and even secondary tumours derived from primary xenografts when they were injected in mice, whereas only one of all injections of ABCB5 negative subpopulation cells formed tumours following the same protocol. This study reported that ABCB5+ subpopulation possesses a higher tumorigenic capacity than ABCB5− subpopulation and it was able to re-establish the clinical tumour heterogeneity when the comparison among tumours was effectuated [21, 28].

Moreover, Schatton et al. found that primary or metastatic melanomas expressed significantly more ABCB5 than benign melanocytic nevi and metastatic melanomas

expressed more to lymph nodes than the primary tumours [21]. Supporting the Schatton et al. results, a recent study observed a higher level of expression of ABCB5 in invasive compared with in situ melanoma and in situ melanoma compared with benign nevi. These data identify ABCB5 as a molecular marker of neoplastic progression [29].

10.3.3 CD 20

CD20 is an activated-glycosylated phosphoprotein expressed on the surface of all B-cells in charge of enabling optimal B-cell immune response, specifically against T-independent antigens [30].

In 2005, Dong Fang et al. reported that a population of melanoma cells had the ability to propagate as non-adherent spheres. When the spheres were separated into individual cells (melanoma spheroid cell) it was found that each cell could differentiate into different cell lineages and persisted after serial cloning in vitro and transplantation in vivo as multipotent melanoma spheroid cells, showing their plasticity and their ability to self-renew. Afterwards it was observed that a subpopulation of melanoma spheroid cells expressed CD20 and it was shown that the CD20+ subpopulation tended to form larger spheres and increased potential for mesenchymal differentiation [20].

10.3.4 CD 271

A marker commonly used for the isolation of stem cells from the neural crest. Malignant melanomas, as normal melanocytes, derive from the neural crest lineage [31]. Also known as nerve growth factor receptor, it is a neurotrophin receptor which can bind all of the neurotrophins by similar affinity and mainly acts promoting cell survival or inducing cell death [32, 33].

In very consistent studies, CD 271 has been shown as a cell subpopulation marker with CSC properties in melanoma as well as a marker able to initiate tumorigenesis, to have the ability to form metastases in experimental animal models in vivo, to sustain long-term tumour growth and to regenerate tumour heterogeneity similar to the primary tumour even after several passages [34, 35]. Civenni et al. found that in primary tumours with an evidence of metastasis and in metastatic lesions, the number of CD271/SOX10 positive cells was relatively increased, which suggests that their frequency is associated with the metastatic potential in human melanoma [35].

A recent study has established a relationship between CD271+ subpopulations and vasculogenic mimicry-forming tumour cells, and at the same time, it has shown that these cells are more resistant to cytotoxic agents [36].

10.3.5 ALDH

Is a polymorphic enzyme responsible for the oxidation of aldehydes to carboxylic acids and it is believed to play a role in the differentiation of stem cells via the metabolism of retinal to retinoic acid [37]. One approach to find markers of tumorigenic cells is to focus on conserved progenitor cell functions and interestingly, the ALDH activity can be used to sort a subpopulation of cells which displays stem cell properties from benign tissue from cancer. It has been observed in a variety of tumours that cells with high ALDH activity (ALDH HIGH) are enriched in cancer stem cells [38–40].

Boonyaratanakornkit et al. isolated in a NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice model the frequency of melanoma initiating cells in melanoma tissue from different patients, finding in all patients that only a fraction of tumour cells initiated further tumor growth. It was shown that human melanoma cells with ALDH HIGH were enriched in tumorigenic cells over unfractioned cells (only 1 in 21,000 cells was a melanoma initiating cell). Afterwards, they xenografted subpopulations of ALDH positive cells and ALDH negative cells to mice subcutaneously and it was reported that ALDH positive subpopulation melanoma cells had enhanced tumorigenicity and superior self-renewal ability over ALDH negative [41].

Santini el al. showed consistent results in the same line, with regard to the high activity of ALDH and their relationship to melanoma CSC, showing that the spheres of melanoma are capable of tumour initiation and have stem cell properties. It was observed that only those tumors which had near to 10 % or a higher percentage of ALDH HIGH cells were capable to form spheres in culture, suggesting a good correlation between ALDH HIGH and efficient formation of spheres. Subsequently ALDH HIGH and ALDH LOW cells were isolated and they were injected into athymic nude mice, reporting that tumours generated from ALDH HIGH were significantly larger and grew faster than those tumours which were originated from ALDH LOW cells [42]. These studies provide an evidence for a phenotypically distinct tumorigenic cell in melanoma which has superior self-renewal and tumorigenic ability.

10.3.6 Nestin

Is an intermediate filament protein originally identified in neuroepithelial stem cells and it is expressed during mammalian embryogenesis in a variety of tissues, but under pathological conditions (i.e. injury to the central nervous) it could be re-expressed in adults [43]. The protein expression is regulated by several transcriptional factors among others Sox [44]. A subpopulation of nestin expressing cells with stem cell properties has been identified in melanoma cell lines by Grichnik et al. showing that nestin positive subpopulation was expressed in cells which had

the ability to maintain the morphologic and antigenic heterogeneity intact even after clonal purification [45].

Initially, in 1994 nestin was observed to be higher expressed in a fraction of melanocytic tumours, particularly the metastatic melanomas, suggesting that it may be a useful marker for melanomas. In this document the authors showed that in the invasive part of the tumours nestin was abundantly expressed indicating, this way, a possible involvement of nestin in tumour infiltration [46]. Supporting these data Brychtova et al. evaluated nestin expression in 139 tissue samples of cutaneous melanoma and melanocytic nevi and demonstrated that nestin expression predominated in lesions with dermal invasion over 1 mm and endothelial nestin overexpression in vessels surrounding advanced melanomas. It was concluded that nestin might be an indicator of tumour dedifferentiation and of more aggressive behavior [47].

Another recent study has determined nestin expression in melanoma tissue and in blood patients with melanoma and it has been found that nestin expression was significantly lower in stage III/IV patients with no evidence of disease than in stage IV and it was significantly higher in patients with high vs. low tumour burden, suggesting that circulating nestin expression reflects the tumour burden closely [48].

10.3.7 SOX Family Transcription Factors

The SOX (Sry-type HMG box) is a family gene code for transcription factors which either induce or suppress lineage-specific genes during embryonic development, and exert their main activities by binding to DNA [49].

A recent study has analyzed the relationship between Sox 10 transcription factor and melanoma, reporting a 100 % expression of Sox 10 in primary melanomas. Afterwards Sox 10 in human melanoma cell lines was silenced by means of RNA interference, which showed a reduction in the capacity to form clones on SOX 10 knockdown cells. When the SOX 10 silenced cells were subcutaneously injected in immunocompromised mice, none of these injections led to tumour formation, whereas 11 out of 14 injections of Sox 10 positive cells produced tumours in vivo, demonstrating the relevance of SOX 10 in tumorigenesis. At the same time, it was reported that a ninefold increase in percentage of apoptotic cells when SOX 10 silenced cells were treated, revealing the important role of SOX 10 in supporting the survival of melanoma cells. The authors concluded that Sox 10 positive cells possess stem cell properties [50].

Bakos et al. [51] demonstrated that SOX 9 and SOX 10 (key regulator of pigment cell formation during embryonic development and expressed in all stages of melanocyte differentiation) [52] are expressed in different melanocytic tumours, showing higher levels of expression especially regarding SOX 10 protein in primary and metastatic melanomas in comparison to nevi, supporting the results obtained by Cook et al. [53] who demonstrated that cultured melanoma cells had 9.5 fold more amounts of SOX 10 than melanocytes.

Some studies have reported a relationship between SOX family transcription factors and nestin. Flamminger et al. recently showed that SOX 9 and SOX 10 are required for nestin expression in human melanoma [54], while Bakos et al. showed statistical significance ($p < 0.05$) co-expression of nestin with SOX 9 and SOX 10 [51]. Similarly Laga et al. reported that SOX 2 and nestin are co-expressed in human melanomas and SOX 2 expression correlates with a different pattern of nestin distribution [55].

10.4 Signaling Pathways in Melanoma Stem Cells

It is known that mesenchymal stem cells, (MSCs) which are responsible for regeneration and cellular homeostasis, are present in almost every tissue. The essential self-renewal controlling pathways (Wnt, Notch and Hedgehog) are conserved in these ubiquitous stem cells. Multiple investigations have demonstrated that Cancer Stem Cells biology is regulated by the same signaling pathways.

10.4.1 Wnt/B-Catetin Pathway

Wnt/beta-catenin pathway is involved in biological processes such us embryogenesis, development, cell polarization, differentiation and proliferation. During embryogenesis, Wnt proteins direct cell fate determination at various stages of development and their signaling acts to regulate the development of a variety of organ systems including cardiovascular, central nervous system, renal and respiratory systems. In adults, Wnt signaling has a key role in the regulation of tissue self-renewal, particularly in intestinal crypts, hair follicles, and bone growth plates.

Wnt are secreted glycoproteins which bind to cell surface receptors to initiate signaling through intracellular molecular cascades. The canonical Wnt pathway is initiated when a Wnt ligand binds Frizzled and low-density lipoprotein receptor-related protein (LRP) families, leading to the stabilization and nuclear translocation of B-catenin, where it forms a complex with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and ultimately driving transcription of target genes including c-myc, c-Jun, cyclin D1 or surviving, genes involved in cell cycle regulation, proliferation and apoptosis inhibition (see Fig. 10.1).

Wnt/beta-catenin pathway is highly conserved in different cell lines, including: embryonic stem cells (ES), brain, pancreatic islet, colon cancer, head and neck tumour, melanoma and pancreatic cancer [56]. It is also conserved in a variety of CSC settings, including colon, breast, brain, cutaneous CSC, and in hematopoietic stem cells [57–62].

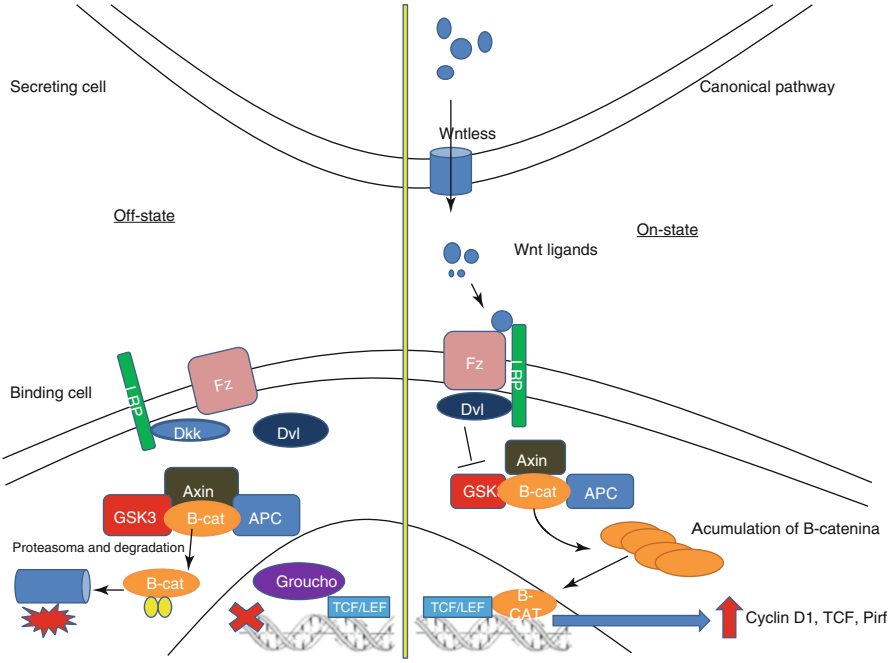

Fig. 10.1 Wnt/beta-catenin pathway: Wnt ligands are released by the sending cell through Wnt less. Signaling pathway is initiated with binding of Wnt ligands to Fz and LRP 5/6 co-receptors forming a complex which initiates the intracellular cascade. This process results in disruption of the beta-catenin destruction complex with the absence of phosphorylation of beta-catenin, accumulation and translocation to nucleus. In nucleus, beta catenin binds to TCF/LEF driving transcription of c-myc, cyclin D, survivin genes involved in cell cycle regulation, proliferation and apoptosis inhibition. *Fz* frizzled, *LRP 5/6* low density lipoprotein receptor-related protein, *Dvl* dishevelled, *Dkk* dickkopf, *APC* adenomatous polyposis coli, *GSK3b* glycogen synthase kinase 3b, *CK1a* casein kinase 1a

The importance of Wnt/beta-catenin pathway in the CSC biology field was studied by Hoffmeyer et al., who found that β-catenin regulated Tert expression, and thereby telomere length, which has a pivotal role in stem cells [63].

There is not much evidence about the involvement of Wnt/b-catenin pathway in melanoma stem cells: Malanchi et al. studied the relationship between cutaneous CSC and Beta-catenin. Two subpopulations of CD34+ (CSC known marker) and CD34− melanoma cells were compared. Analysing tumorigenic capacity of CD34+ cells, it was checked that the CD34+ population is over 100-fold more potent in initiating secondary tumours than the CD34− cells. In addition, secondary tumours maintain a stable population of CD34+ cells which retain tumour initiation potential, giving rise to tertiary tumours. Secondary and tertiary tumours derived from CD34+ cells closely resembled the architecture of the parental tumour. It was found that nuclear b-catenin expression was enriched in CD34 positive cells versus CD34 negative cells. It was also shown that the deletion of b-catenin in established tumours

led to a reduction of the percentage of CD34+ cells, and the tumours regressed, which suggested a potential functional relevance for this pathway in melanoma stem cells [59].

10.4.2 Hedgehog-GLI Pathway

Hh signaling is a conserved pathway in vertebrates and highly active during mammalian development, especially within the neural tube and skeleton, but subsequently silenced in most adult tissues. However, some post-natal organs, such as the central nervous system (CNS) and the lung, rely on continued Hh signaling for tissue homeostasis and repair following injury.

Hh ligands IHg, DHh, SHh (Indian Hedgehog, Dessert Hedgehog, Sonic Hedgehog) are released from the secreting cell through a dedicated transmembrane transporter, Dispatched, after acylation of Hh N-terminus by the enzyme Rasp located in the endoplasmic reticulum. Binding of Hh to the transmembrane receptor Ptch 1 (Patched) initiates signaling via the Hh pathway. Ptch1 inhibits the Smoothened (Smo) receptor by preventing its localization to the primary cilium, a non-motile projection which is present in most vertebrate cells. In the presence of Hh, the Hh-Ptch1 complex is internalized, allowing Smo activation. Localization of Smo to the primary cilium, instead of to the plasma membrane, initiates a signaling cascade in mammals, leading to the activation of the Gli family of zinc-finger transcription factors. In vertebrates, three Gli proteins are observed: Gli1 serves to activate Hh target genes, Gli2 acts as an activator as well as a repressor, and Gli3 acts as a repressor of target-gene transcription. Hh signaling seems to be dependent on the relative balance of Gli activator and repressor forms. These factors activate the transcription of genes which promote cellular proliferation (see Fig. 10.2).

Recent data demonstrate the importance of Hedgehog-GLI in the CSC biology of many human tumours including glioblastoma, breast cancer, pancreatic adenocarcinoma, multiple mieloma and chronic myeloid leukemia (CML) [64–70]. In mouse models of CML, the loss of Hh signaling by genetically disrupting Smo, inhibited the expansion of BCR-ABL positive leukemic stem cells and prolonged survival [18, 19]. Hh Pathway inhibition with cyclopamine or siRNA produced the loss of tumorigenic potential, in glioblastoma CSCs [71, 72].

Only two studies have analyzed the relationship between Hedgehog-GLI and melanoma stem cells. In 2012, Santini et al. studied a collection of human melanomas obtained from a broad spectrum of sites and stages. It was shown that human melanoma cell lines contained a subpopulation of cells with high ALDH (CSC known marker) activity (ALDH high) and another subpopulation with low ALDH activity (ALDH low). It was concluded that ALDH high cells had more clonogenicity in vitro and tumorigenicity in vivo in comparison with ALDH low cells. The activation of Hedgehog-GLI of both subpopulations was also analyzed. It was found that ALDH high cells expressed a higher level of GLI1 than the ALDH low population, so ALDH high is associated with high Hedgehog-GLI activity. Pharmacological

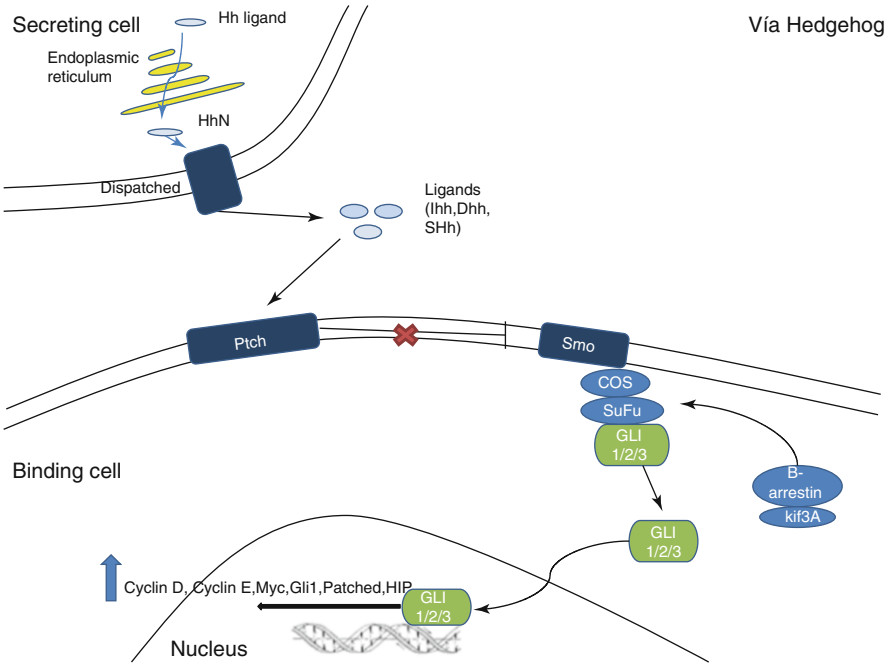

Fig. 10.2 Hedgehog pathway: Hh ligands suffer an acylation of N-terminus in the sending cell. After this, Hh ligands are released from the sending cell through Dispatched. Ligands bind to Ptch which stops the inhibition over Smo. Smo initiates an intracellular signaling cascade which leads to the activation of GLI zinc transcription factors (GLI1, GLI2, GLI3) and to the transcription of target genes, including Cyclin D, Cyclin E, Myc, GLI1, Ptch, HIP involved in processes such as patterning maintenance, stem-cell properties maintenance or tissue polarity. *Ptch* patched, *Smo* smoothened

inhibition of Hedgehog-GLI by the Smoothened antagonist cyclopamine and GLI antagonist GANT61 and stable expression of shRNA targeting either SMO or GLI1 resulted in a significant decrease in melanoma stem cell self-renewal in vitro and a reduction in the number of ALDH high melanoma stem cells. The authors concluded that Hedgehog-GLI pathway plays an important role in controlling self-renewal and tumour initiation of melanoma stem cells [42].

Pandolfi and colleagues analyzed the implication of Hedgehog-GLI in controlling CSC self-renewal, using melanoma CSC and breast CSC cultures (melanomaspheres and mammospheres). The activity of the pathway with shWIP1 (WIP1 is a nuclear phosphatase expressed at low levels in most normal tissues which positively modulates GLI1 activity) and/or shPTCH1 was modified, and the self-renewal capacity in vitro was measured, which allowed the quantification of the ability of dissociated single stem cells to generate secondary spheres. Silencing of PTCH1 increased by twofold the number of melanomaspheres and by ninefold the number of mammospheres compared with control. Silencing of WIP1 slightly decreased the number of melanomaspheres but the number of mammospheres was not changed; however,

it reversed the increase in self-renewal induced by shPTCH1 in both cell types. This data corroborated that the activation of Hedgehog pathway enhances self-renewal capacity in melanoma and breast stem cells [73].

10.4.3 Notch Pathway

Notch signaling has a critical role in the regulation of cell-to-cell communication during embryogenesis, cellular proliferation, differentiation, and apoptosis. Notch signaling is also essential for normal hematopoiesis, breast development, colorectal epithelial maturation, immune regulation, and neural stem cell survival [74, 75].

There are four human Notch receptors which consist of an extracellular peptide which contains epidermal growth factor receptor-like repeats and a transmembrane peptide. Notch 1 and Notch 2 are the most ubiquitously distributed receptors whereas Notch 3 and Notch 4 are more specifically expressed in vascular smooth muscle and endothelial cells. Notch signaling is initiated by a receptor ligand interaction between two neighboring cells via the Jagged (JAG1,JAG2) or Delta-like family of membrane proteins which leads to successive proteolytic cleavage reactions that liberate the cytoplasmic domain of Notch (NICD, Notch intracellular domain) from the membrane. The proteolytic cleavage is mediated by the gamma-secretase activity of presenilin protein complexes. The liberated NICD translocates to the nucleus where it is associated with the transcription factor RBP-J, which turns the RBP-J complex from a transcriptional repressor to a transcriptional activator, from which Mastermind and histone acetyltransferases are recruited as co-activators. The most well-defined targets of the NICD-RBP-J complex are the HES family and their homologous, the Hey (also called HERP) family of basic helix-loop-helix transcription factors involved in maintenance of stem cells, cell fate specification, differentiation, proliferation and apoptosis (see Fig. 10.3).

Notch pathway has been linked to the biology of the CSC from multiple sources: breast cancer [76–80], embryonal brain tumours [81], and gliomas [82, 83]. Farnie et al. demonstrated that Gamma secretase inhibitors (GSIs) abolish the formation of mammospheres from a variety of human breast cancer cell lines as well as primary patient specimens [79]. Fan et al. showed that Notch inhibition selectively depletes medulloblastoma CSC as determined by CD133-high status or dye exclusion [81]. Current evidence of the importance of the Notch pathway in melanoma stem cells is limited, but it is known that Notch and its ligands are abundantly expressed in the epidermis, where Notch signaling functions as a molecular switch which intervenes in cell transition between different skin layers during the epidermal differentiation process [84–86]. Some homeostasis of hair pigmentation studies have demonstrated that the deletion of Notch1 and Notch2 or RBP-Jkappa in the melanocyte lineage results in a severe defect in hair pigmentation, due to the melanoblasts and melanocyte stem cells elimination [87, 88].

Multiple studies have suggested the importance of Notch pathway in melanoma development. Massi et al., showed that the expression of Notch1, Notch2 and their ligands is upregulated in melanomas and ‘dysplastic nevi’ when compared to

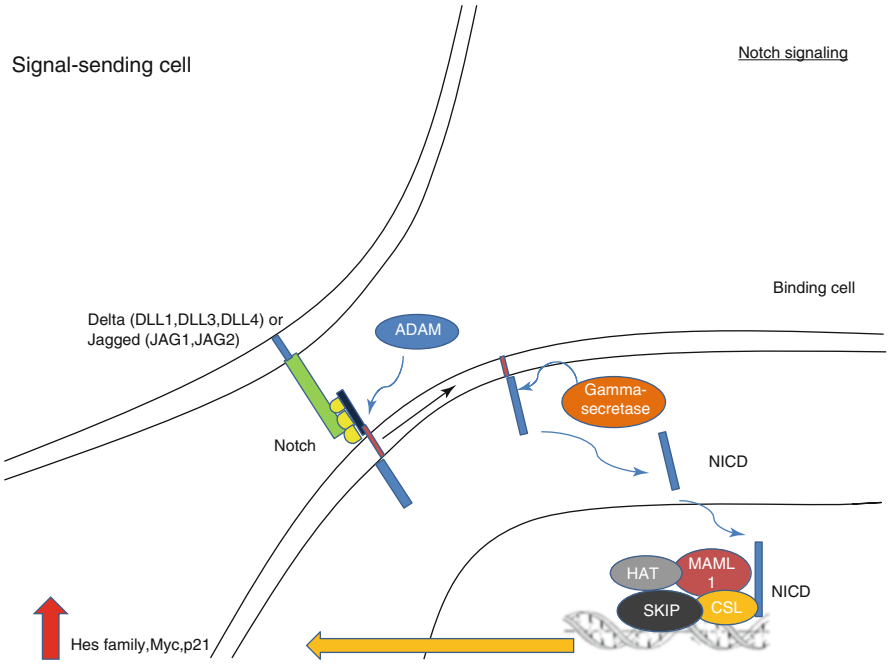

Fig. 10.3 Notch pathway: Activation of Notch pathway occurs between two neighboring cells by binding Delta (DLL1, DLL3, DLL4) or Jagged (JAG1, JAG2) to Notch receptor. Then, ADAM/TACE produces the proteolytic clivage of extracellular domain of Notch. Gamma secretase does the same with NICD, which is released to the cytoplasm and is translocated to the nucleus where it serves as a transcriptional activator of target genes like HES, Myc, p21 involved in maintenance of stem cells, cell fate specification, differentiation, proliferation and apoptosis. *ADAM/TACE* A disintegrin and metalloproteinase, *NICD* intracellular domain of Notch

common melanocytic nevi [89]. Hoek and his colleagues found similar results detecting Notch2 mRNA overexpression in melanoma cells compared to nevi and normal melanocytes [12].

Balint et al. demonstrated that melanoma cell lines acquired an enhanced metastatic ability when the Notch1 pathway was constitutively activated, and also suggested that this effect was mediated by ß-catenin, as beta-catenin was upregulated following Notch1 activation [90]. It was also found that inhibition of γ-secretase in melanoma cell lines induced apoptosis, but not in normal melanocytes, which suggests that Notch is required for melanoma cell survival [90, 91].

10.5 Prognostic Relevance of Melanoma Stem Cells

Cancer Stem Cells are characterized by their ability to self-renew and to establish tumours upon transplantation, remain quiescent for long time and for their innate resistance to chemotherapy and radiotherapy. These features suggest that they are responsible for relapse and metastasis.

Multiple studies have examined the prognostic value of various markers of melanoma CSC, concluding that overexpression of these molecules correlates with a worse prognosis. The most important prognostic markers will be reviewed along this section.

10.5.1 Prominin-1 (CD133)

The relationship between CD133 expression and prognosis was first documented in 2010. Al Dhaybi et al. analyzed the CD133 expression in childhood malignant melanoma. The expression of CD133 and Ki67 was evaluated in 12 cases of malignant melanoma and 12 control cases of Spitz nevi, and it was found that only the three cases with lymph node metastasis and the only case with visceral metastasis were positive for CD133. None of the Spitz nevi had positive expression of CD133. The CD133-positive cells had a lower Ki-67 index than the CD133-negative cells, which might explain their chemoresistance [92]. Similar results in adults were published by Sharma and colleges, who found a significant overexpression of CD133 ($p<0.02$) by inmunohistochemical staining in tissues from patients with recurrent disease versus those without disease recurrence. Relative risk analysis between these two groups suggested that the patients with recurrence or metastatic lesions had a greater than twofold overexpression of CD133. To investigate the potential of CD 133 as a molecular biomarker of melanoma progression and disease recurrence, CD133 mRNA transcript levels were assessed by qRT-PCR in cell lines from fresh tissues of patients with poor outcome and short overall survival as well as from fresh tissues of patients with good outcome and long overall survival status. CD133+ mRNA transcripts were expressed at levels 15–20 times higher in the latter group, suggesting that CD133 transcripts strongly and negatively correlated with the clinical outcome, and were thus a potential predictor of poor prognosis in high-risk melanoma ($p<0.04$) [16].

Piras et al., published that a higher than 10 % CD133 expression in endothelial cells of the tumor mass detected in nodal metastasis is significantly associated with poor survival ($p=0.008$) [17].

10.5.2 Nestin

Nestin is one of the stem cell markers which has been more commonly associated with prognosis in melanoma. After the document published by Klein et al., which demonstrated that primary and metastatic melanoma significantly expressed higher levels of CD133, CD166 and nestin than banal nevi [18], Brychtova and colleges, suggested that expression levels of nestin may be a marker of tumour aggressiveness, as it was found that nestin expression was increased in malignant melanomas in respect to melanocytic nevi, specially in ulcerated melanomas, and its levels correlated with the clinical stage of tumour [47].

Subsequently, Piras et al., published an inmunohistochemical study for nestin and CD133, performed in 130 primary melanomas and 32 nodal metastasis biopsy specimens, where their expression and their correlation with survival data and clinicopathological variables were evaluated. Nestin expression in cytoplasm of tumoral non-pigmented cells and endothelial cells was observed. This expression was more numerous at the invading tumour front of primary tumors and in the peripheral areas of the multiple small nodules within the tumor mass. The Kaplan-Meier analysis showed that nestin expression higher than 10 % of cells, significantly predicted poor survival in patients with stage I and II melanoma (global $p=0.037$). The best group (those with no nestin expression in tumoral non-pigmented or endothelial cells) in comparison with the worst (fully positive tumours) had a significant better outcome with a 86.2 % vs. 58.8 % of 5-year survival rate. In stage IV melanoma, a high significant correlation between the presence of nestin in tumoral cells ($p=0.006$) or in vessels ($p=0.034$) of nodal metastasis and survival was reported. Nestin had a higher predictive value when its expression in tumoral cells and vessels was studied in combination ($p=0.005$) [17].

Likewise, Tanake et al. analyzed 78 malignant melanomas for nestin, HMB-45 and S100 inmunohistochemical expression, and found that nestin was detected in 56.5 % of malignant melanomas. The prognostic relevance of nestin expression was analyzed and a significant difference in survival rate among the stages was observed; moreover, the 5 years survival rate of stages I and II nestin-positive cases was significantly decreased compared to the nestin-negative patients ($p<0.05$). In addition, the 5-year survival rate was 100 % in all patients exhibiting nestin-negative malignant melanomas at all stages of tumour development. The authors concluded that the nestin expression may be a predictor of poor prognosis in patients with malignant melanoma [93]. On the other hand, Fusi et al. characterized melanoma cells circulating in blood from patients with metastatic melanoma for expression of stem cell-related markers, CD133 and Nestin. Assuming that metastasis requires a dissemination of tumor-initiating cells, the authors posed that the identification of these cells expressing CD133 and nestin in peripheral blood should be associated with worse patient outcome. It was found that nestin expression (positive CMCs >35 %) correlated with poor outcome ($p=0.006$), as well as with levels of LDH, number of metastatic sites, and tumour burden. But the positivity of circulating melanoma cells (CMCs) for CD133 was not associated with overall survival [94].

10.5.3 ABCB5

Schatton and colleagues identified a subpopulation which was enriched for human malignant-melanoma-initiating cells, defined by expression of the chemoresistance mediator ABCB5 [21]. And Setia el al. observed a higher level of expression of ABCB5 in invasive compared with in situ melanoma, and in situ melanoma compared with benign nevi, which might indicated that ABCB5 is a key player in melanomagenesis [29].

ABCB5 is a member of the ABC transporter family and it plays a role in drug efflux, participating to chemoresistance. In 2012, Chartrain and colleagues explored the effect of anti-melanoma treatments on the ABCB5-expressing cells. In a xenograft model, it was demonstrated that ABCB5-expressing cells selectively survive over ABCB5− cells after a Temozolomide treatment inducing a significant tumour regression. The expression of ABCB5 in human melanoma metastatic samples obtained before and after Dacarbazine treatment was also analyzed, and it was found that the drug was associated with a selective survival and an increased number of cells which expresses ABCB5 protein on their surface. In vitro, it was showed that ABCB5-expressing cells selectively survive when exposed to Dacarbazine, but also to Vemurafenib, an inhibitor of V600E BRAF and other various chemotherapeutic drugs. The authors concluded that the chemoresistance acquisition which leads to clinical relapse is mediated by the selection of tumour cell subpopulations such as ABCB5-expressing cells, due to chemotherapeutic agents [95].

Using multimarker quantitative real-time polymerase chain reaction (qRT-PCR) for detecting circulating tumour cells in the peripheral blood of patients with melanoma, Reid and colleagues investigated whether the phenotype of circulating melanoma cells could represent a useful indicator of disease stage, recurrence and treatment efficacy. Peripheral blood from 230 patients (154 with stages I-II and 76 with stages III-IV melanoma) was analyzed and 152 healthy controls, using qRT-PCR analysis for the expression of five markers: MLANA, ABCB5, TGFβ2, PAX3d and MCAM were effectuated to find that MLANA and ABCB5 expression correlated with disease stage, with expression more common in advanced (stage III–IV) than in early-stage (stage 0–II) patients. It was also demonstrated that MLANA and ABCB5 had significant prognostic value, as they were identified as statistically significant among patients who experienced disease recurrence, being expressed in 45 % (MLANA) and 49 % (ABCB5) of patients with recurrence (P = 0·001 and P = 0·031, respectively). Moreover, it was found that ABCB5 was detected among patients regardless of whether or not they were considered clinically disease free, indicating that these stem-like circulating cells remain for long periods in the blood. The study concluded that MLANA and ABCB5 expression in blood could be a potential predictor of disease recurrence or progression [96].

10.5.4 Other Melanoma Stem Cells Biomarkers Related to Prognosis

Civenni et al. analyzed the expression of neural cancer stem cells markers, ***Sox10*** and ***CD271***, in 200 biopsies of primary melanomas, melanoma metastasis and melanoma cell lines, and found that the proportion of CD271/Sox10 double-positive cells in primary tumours without any evidence of metastasis was significantly less than in primary melanomas, which developed metastases during the 5 years follow-up (p 0.01). In addition, there was a higher proportion of CD271/Sox10 positive

cells in metastasis compared with primary melanomas without metastasis (p 0.04). The analysis was then focused on 54 primary melanoma of a sentinel lymph node study where tumor-specific survival was available; the frequency of greater than 5 % CD271/Sox10 positive cells was associated with poor tumour-specific survival (p 0.03). As a result of these findings, the authors concluded that an elevated frequency of melanoma cells expressing neural CSC markers is a prognosis factor for the development of metastasis [35].

B-cell-specific Moloney murine leukaemia virus Integration site 1 (BMI-1) is a transcriptional repressor of the Ink4a/Arf locus encoding p16 (ink4a) and p14 (Arf), two separated tumor suppressor genes. BMI-1 is highly expressed in embryonic stem cells and the placenta. BMI-1 has been recently shown to play a crucial role in self-renewal of stem cells because of its function in repressing senescence and cell death [97, 98].

Mihic-Probst et al. studied the expression of stem cell markers BMI-1, p16/ink4a and nestin in 64 cutaneous melanomas, 165 melanoma metastasis and 53 melanoma cell lines, and demonstrated that high BMI-1 and low p16/ink4a expression are predictors of metastatic disease (p 0.02 and 0.04; respectively). The cases with high BMI-1 and low p16/ink4a had an even higher risk of lymph node metastasis (p 0.005). In multivariate logistic regression analysis, high BMI-1 expression was an independent predictor of metastatic disease [99].

10.6 New Therapeutic Strategies Targeting Melanoma Stem Cells

Cancer Stem Cells received increasing attention as novel targets for cancer therapy; in particular as an emerging evidence, it indicates that CSCs are substantially associated with tumor initiation, angiogenesis, cancer maintenance and metastasis. These cells have an innate resistance to chemotherapy and radiation, and are postulated as responsible of treatment failure and relapse. Many possible ways were developed to eradicate CSCs, and to improve survival or to cure cancer, including molecular targeted therapy, target molecular signaling pathways, differentiation therapy, and natural compounds and their potent to target CSCs [100].

Preclinical studies about ***stem cell biomarkers*** were used for isolation of melanoma CSCs and also showed that its blockade could serve as antitumor treatment. Rappa et al. [101] investigated the effects of CD133 downregulation in vitro and in vivo in human metastatic melanoma. Downregulation of CD133 resulted in slower cell growth, reduced cell motility, and decreased capacity to form spheroids under stem cell-like growth conditions. Aldehyde deshydrogenase (ALDH) is a polymorphic enzyme responsible for the oxidation of aldheydes to carboxylic acids. Luo et al. [102] demonstrated the existence of human melanoma cells that fulfill the criteria for CSCs by serially xenotransplating cells into mice, which possessed high ALDH activity, with ALDH1A1 and ALDH1A3 being the predominant ALDH

isozymes. Silencing ALDH1A1 by siRNA or shRNA leads to cell cycle arrest, apoptosis, decreased cell viability in vitro and reduce tumorogenesis in vivo. Moreover, many authors consider that the blockade of these stem cells biomarkers, by antibodies or by other means, could lead to severe side effects, given that they are usually broadly expressed in healthy tissue.

Drug discovery approaches to target crucial ***signaling pathways*** in cancer stem cells including the Wnt, Notch and Hedgehog signaling pathways are currently explored at different levels. No studies with signaling pathways inhibitory molecules have been conduced in melanoma. Relevant published results and data concerning other tumors would open up new mono-therapeutic or combinatorial therapeutic possibilities and could support their use. Inhibition of Wnt/β-Catenin pathway has been explored in colorectal cancer with COX2 inhibitors and natural compounds like vitamin A and D and their derivates [103]. Notch signaling pathway plays a critical role in the maintenance of CSCs and its inhibition has been studied in glioblastoma and breast cancer [104–106], above all. It was suggested that overexpression of Hedgehog (Hh) is active by NF-κB in pancreatic cancer and pancreatic cancer cell proliferation is accelerated, in spite of this fact Hh pathways inhibitors have successfully been investigated in pancreatic CSCs [100].

Differentiation therapy is an important approach to the treatment of advanced or aggressive malignancies. There are several methods which could be used to induce cancer stem cells differentiation. Vitamin A and its analogue (retinoid) can reverse the malignant progression process through signal modulations mediated by nuclear retinoid receptors and all-trans retinoid acid produces remission of acute promyelocytic leukemia by inducing promyelocyte differentiation [107]. The new differentiation-inducting agents are represented by those ligands which can induce stem cells to undergo asymmetric mitosis in various cancer types. Those agents would include gene products of Wnt, Hedgehog, TGF and EGF and can be delivered to the CSCs to force them to switch from a symmetric to an asymmetric mitotic program. On the other hand, the use of antisense inhibitors or ribozyme agents which block specific factors, which inhibits asymmetric mitosis or activates symmetric mitosis, could cause asymmetric cancer stem cell line mitosis [108].

Over the last few years, several studies have demonstrated the possibility that certain ***natural compounds*** have to attack CSCs and their ability to target cancer stem cell. Salinomycin, a polyester antibiotic which acts as a highly selective potassium ionophore and widely used as an anticoccidial drug, operates as a potent inhibitor of multidrug resistance P-glucoprotein (P-gp 170). It was shown to function as a specific inhibitor of CSCs in several studies in human lung adenocarcinoma, with down-regulated expression of stem cells markers OCT4, NANOG y SOX2 involved in blocking self-renewal and proliferation [109]. Also, its effects have been studied in ovarian cancer and lymphoblastic leukemia cells. Curcumin is present in Indian spices and possesses anti-inflammatory and antioxidant activities. It acts as a modulator of ABCG2 and it can also act on ABCB1 and ABCC1. This mechanism has been studied in the side population phenotype of the rat C6 glioma cell line [110] and in all tested cancer cell line including gastric, colon and intestinal cancer cells and its efficacy has been demonstrated [111]. Marine sponge extract has been

identified in marine environment and it has characteristics by affecting self-renewal potential, apoptosis resistance, invasive potential and tumorigenicity in mice and its effects have been tested in pancreatic and prostate CSCs [112]. Sulforaphane has demonstrated to eliminate pancreatic CSCs by down regulation of NFKB activity with minimal side effects, in combination with Sorafenib [113]. Several studies have reported the activity of sulforaphane of down-regulation Akt pathway in ovarian, prostate and colorectal cancer [114, 115] and recently, PI3K/Akt pathway have demonstrated to play an important role in regulation breast stem cells by promoting Catenin downstream events [116]. No studies related to these molecules have been conduced in melanoma.

10.6.1 Preclinical Data in Melanoma CSCs

The benefit of ***radioimmunotherapy*** (RIT) in melanoma CSCs is reported in several studies. The first Phase Ia/Ib clinical trial of 188Re-6D2 mAb in patients with Stage III/IV melanoma demonstrated that safety of RIT was indicative of its efficacy and prolonged patients median survival [117]. In a preclinical study [118], encouraged by the result of first clinical trial of melanin-binding mAB, it is reported the mechanism underlying the efficacy of melanoma RIT in relation of melanoma CSCs. Mice bearing A2058 melanoma xenografts were treated with either 1.5 mCi 188Re-D2 antibody saline, unlabeled 6D2 antibody or 188Re-labeled non specific IgM. On day 28, post-treatment tumor size in the RIT group was four-times less than in control group ($p<0.001$). Two melanoma CSCs markers-chemoresistance mediator ABCB5 and H3K4 demethylase JARID1B were used to analyze tumors by immunohistochemistry and FACS. Significant differences between RIT and control groups in percentage of ABCB5 or JARID 1B positive cells in the tumor population were not found. The results of the investigation of the RIT effects on ABCB5+ and JARID1B+ melanoma CSCs during the RIT of experimental human melanoma in mice with radiolabeled 6D2 mAb to melanin were reported and melanoma CSCs were demonstrated not to have elevated resistance to RIT in comparison with the rest of tumor cells. The study results demonstrated two main implications for melanoma treatment. Firstly, the susceptibility of ABCB5+ and JARID1B+ cells to RIT. Secondly, specifically targeting cancer stem cells with radiolabeled antibodies to ABCB5 and JARID1B might help to completely eradicate cancer stem cells in various cancers.

MicroRNAs is an attractive therapeutic tool to revert tumor proliferation [119]. Melanoma CSCs have been isolated with CD133, CD44, ABCB5 marker or side population (SP) phenotype. The inhibition of CD133 expression by short hairpin RNA (shRNA) in human melanoma cells reduce proliferation and cellular migrations in vitro. In vivo, when intravenous tumor cell inoculation of the corresponding shRNA occurs, CD133 downregulation inhibits the development of pulmonary and spinal metastases [101]. Monoclonal antibodies or small inhibitory RNA (siRNA)-mediates ABCB5 inhibition can sensitise melanoma cells to die by chemotherapeutic

drugs [19]. Tumor microenvironment, as an important factor of carcinogenesis, is downregulated by miR-27b when human melanoma cells are exposed to the human stem cell microenvironment [120].

10.6.2 Clinical Trials Targeting Melanoma CSCs

In a recently study, ***therapeutic anti-CD20 antibody*** Rituximab produced regression of melanoma metastases [121]. Although CSCs have similar functional capacities, they don't always share a common marker. In the melanoma CD20 was first reported to be expressed on CSCs [20]. The authors reported the effect of off-label use of local treatment with therapeutic anti-CD20 antibody Rituximab in a patient with metastatic melanoma who had been progressed on multiple lines of treatment. Data of this trial in a patient with metastatic melanoma provided the first evidence of the fact that targeting the minor subset of CD20+ "melanoma sustaining cells" produces regression of chemotherapy–refractory melanoma. Based on preclinical data, it indicated that melanoma is maintained by minor subset of cancer cells characterized by CD20 expression, the patient was treated with lesional injections of very low doses of the anti-CD20 therapeutic antibody Rituximab and concomitant Dacarbazine systemic treatment. Although the frequency of melanoma cells within the tumor lesion was initially about 2 %, Rituximab treatment produced lasting remission as well as a decline of the melanoma serum marker S-100 to physiological levels. Study results reported that local treatment with anti-CD20 antibody Rituximab produced complete regression of all melanoma metastases with the exception of one lesion which only produced partial but stable regression. Study results highlight the potency of selective cancer cell targeting in the treatment of melanoma of a greater patient cohort.

For many years, an active specific ***immunotherapy with autologous tumor antigens*** has recognized a promising immunotherapy approach for patient with metastatic melanoma, in order to potentially consolidate other treatment modalities. In the first trial [122], 74 metastatic melanoma patients, who were treated with subcutaneous (SC) injections of tumor cells weekly for 3 weeks and then monthly for 5 months. The median survival was of 20.5 months and 5-year survival was 29 %.

In a subsequent trial [123] metastatic melanoma patients were treated with SC injections of dendritic cells (DC) loaded with antigens of autologous tumor cells, suspended in GM-CSF, again treated weekly for 3 weeks and monthly for 5 months; 50 % were alive 5 years later. Many patients, who previously had relapsed shortly after variety of therapies, subsequently enjoyed long interval of progression free survival after treatment with DC vaccine [124].

Both approaches are intended to induce, or enhance, B-cell and T-cell antitumor activity through DC antigen presentation, but the data were unconvincing because of the limitations of such historical comparisons. A recent randomized phase II trial of dendritic cells (DC) versus tumor cells in patient with metastatic melanoma [125] reported that immunotherapy with DC vaccine is associated with longer survival

compared with a tumor cell vaccine. Forty two patients were randomized to receive irradiated autologous proliferating tumor cells or autologous dendritic cells (DC) loaded with antigens from such cells. There is increasing evidence that such continuous cell cultures are enriched for self-replicating tumor stem cells and/or early progenitor cells which are able to initiate new sites of metastatic cancer. Both products were injected subcutaneously in 500 μg of granulocyte-macrophage colony stimulating factor, weekly for 3 weeks and then monthly for 5 months. Patients in the two arms did not differ in baseline characteristics. Treatment was well tolerated. At the time of initial analysis, with no patients lost to follow-up, 50 % of patients deceased, and all patients followed for at least 6 months after randomization, survival is superior in DC arm (HR = 0.27; 95 % confidence interval, 0.098–0.729), with a median survival not reached versus 15.9 months, and 2-year survival rates of 72 % versus 31 % (P = 0.007). The obvious limitations of this study at the time of analysis are the small size and incomplete follow-up; however the results are consistent with previous data suggesting a survival benefit from this patient-specific immunotherapy.

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Chapter 11
Cancer Stem Cells in Brain Tumors

Fernando Hurtado de Mendoza and Enrique Alanya Rodriguez

Abstract Some small-sized studies have suggested that CD133 expression and the ability for neurosphere formation have prognostic value in glioblastomas. In a large scale expression study, human glioblastomas were grouped in proliferative, proneural, and mesenchymal tumors. Neural stem cell markers, including CD133 and the formation of neurospheres were upregulated in molecular proliferative subtypes that correlate with a poor prognosis. Thus, CD133 expression and the formation of tumorspheres are completely absent in secondary glioblastomas, which are histologically similar, but different from a molecular point of view with respect to primary glioblastomas. Anaplastic oligodendrogliomas, oligoastrocytomas and glioblastomas with an oligodendroglial component are high grade oligodendroglial tumors, which are difficult to classify because of intratumoral diversity and the absence of clear cut histological markers. It is known that the frequency of tumor sphere growth and a CD133(+) population in high grade oligodendroglial tumors is related with a poor prognosis. Taken together, the presence of CD133(+) stem cells or cell populations with other stem cell biomarkers, and the frequency of tumor sphere formation may become a useful criterion for predicting the response to therapy and for establishing new prognosis glioma subtypes.

Keywords Brain tumors • Cancer stem cells • Glioblastoma • Hedhehog • Notch • Transforming-growth factor • Wnt

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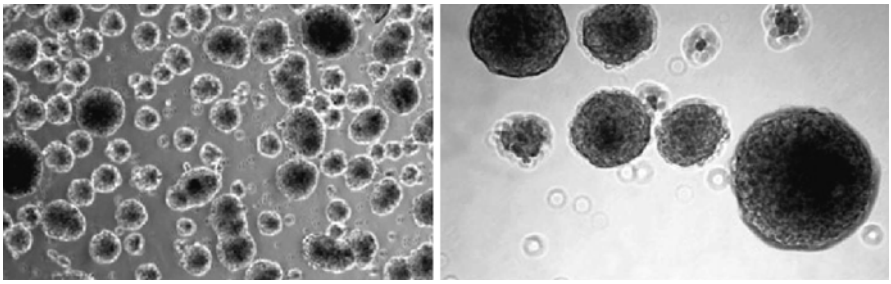

Fig. 11.1 Microphotograph showing high-density neurospheres in culture, 4×(*left*) and 10×(*right*) magnification

11.1 Brain Tumor-Initiating Cells and Their Discovery

The concept of neoplastic stem cells has suggested that tumors are organized within a hierarchy with different cell clone populations that have different proliferative potential [1]. Therefore, it is crucial to characterize the existence of these cells by showing their proliferation, self-renewal and differentiation properties in vitro. Furthermore, since the only real measure of a neoplastic stem cell corresponds to its capacity for generating an equal copy of the tumor from which it is derived, in vivo validation is quite a challenge [2, 3]. With the use of assays developed by Uchida and Buck for purifying neural stem cells with the use of microspheres, Singh et al. reported the identification and purification of a cell from a human primary cerebral tumor with a different phenotype with marked proliferation, self-renewal, and differentiation capabilities [4] (Fig. 11.1).

These cells represent a small proportion of the tumor cell population, and they were identified because of the expression of the CD133 surface marker. These CD133-positive cells, which were then named brain tumor stem cells, lacked the expression of neural differentiation markers and they were considered as necessary for tumor proliferation and self-renewal in cultures. These cells were also found to be capable of differentiating –in vitro– in cellular phenotypes identical to those from the tumor in situ. Independently of the tumor type, the marker phenotype of the brain tumor stem cells was similar to that of the normal neural stem cells, both expressing CD133 and nestin. This suggests that brain tumors may be generated from tumoral stem cells that share a very similar phenotype. The discovery of stem cells for cancer in human gliomas widens the definition of brain tumor stem cells aiming to describe a cellular type that may be able to rule tumorigenesis in a growing number of brain tumors, both in pediatric and adult subjects [5].

11.2 Isolation of Brain Tumor Stem Cells in Tumorspheres

Brain tumor stem cells were isolated for the first time because of their ability to grow forming spheroid structures called tumorspheres under non adhesive conditions. This technique was originally used for isolating neural stem cells from different

areas in both the adult and developing brain, based in the formation of large adherent clones and non-adherent spheroid structures which correspond to neurospheres. In response to conditions with absence of serum, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) [6–8], both adult and embryonic neural stem cells grew as neurospheres, preserving their ability to undergo extensive self-renewal and for differentiating into multiple brain cell types. The clonal neurosphere assay proved to be quite useful in the isolation and characterization of tumor stem cells from pediatric anaplastic astrocytomas and glioblastomas, as well as from adult oligodendrogliomas, glioblastomas, and anaplastic astrocytomas, in a retrospective mode.

An internal cell subpopulation dissociated themselves into tumorspheres capable of forming high-grade gliomas, while the remaining cells showed adherence, loss of proliferation and subsequent differentiation under (appropriate) conditions for the formation of neurospheres. It is important to notice that tumorspheres showed extensive self-renewal and proliferation compared to control neurospheres [4], and they generated a large enough number of descendants that may be capable of becoming differentiated when growth factors in astrocytes, oligodendrocytes, and neurons are taken out [9]. High grade astrocytic tumors predominantly express a set of characteristic markers for glial progenitor cells, neural progenitor cells, and mature astrocytes, while mature neurons and oligodendrocytes are extremely rarely found in high grade gliomas [10, 11]. Tumorspheres from human astrocyte tumors are variably differentiated in GFAP(+) astrocyte type cells and they rarely become GalC(+) oligodendroglial cells in vitro [4, 9]. Thus, in spite of their multipotential ability, tumorspheres preserve the somewhat restricted and heterogeneous potential for aberrant differentiation found in human gliomas [12]. Although there is a variable potential for differentiation between individual high-grade tumors, clonally derived tumorspheres from a tumor give rise to similar proportions of glial cells and neurons. Taken together, these observations suggest that the stem cell population within a given tumor is homogeneous. In summary, the formation of tumorspheres allows the preservation of the unique malignant features from tumor inducing cells, as well as the observed heterogeneity between tumor initiating cells in different malignant conditions.

11.3 Surface Markers in Brain Tumor Stem Cells

Phenotypic cell isolation assays based on surface proteins have been adapted aiming to separate small subpopulations of tumor cells. Human gliomas and microspheres derived from such tumors variably express genes related to stem cells. The surface cell marker CD133 is particularly interesting, being the human counterpart of a protein called prominin-1 (PROM-1). Most variants of PROM-1/CD133 are widely expressed by brain cells [13]. The expression of a variant of PROM-1/CD133, which is recognized by the AC-133 antibody, is more restricted to immature cells, and it has been used for the isolation of neuroepithelial progenitor cells, embryonic neural stem cells from the ventricular areas and from the postnatal cerebellum, as well as from brain tumor stem cells from adult glioblastomas. In 2008, Yang et al.

observed that tumors in murine models of neuroblastoma, using patched mutant mice, are propagated not by CD133(+) neural stem cells, but by cells expressing Math 1 and CD15/LeX progenitor markers [14]. Cells positive for both CD15/LeX and Math1 have a greater proliferative capability, but their apoptosis and differentiation are reduced [15]. CD15/LeX (+) cells are also found in a subtype of human medulloblastoma with a poor prognosis. These data suggest that PROM-1/CD133(+) neoplastic stem cells and tumor propagating cells may be different; and that some human tumors may be propagated by a progenitor-type tumor cell population. Not all neoplastic stem cells are PROM-1/CD133(+), and the expression of PROM-1/CD133 is downregulated in neural stem cells in the subventricular zone in adult subjects [16, 17]. The origin of PROM-1/CD133(+) cells in human glioblastoma tumors has yet to be determined. One possibility is that these may originate from neural stem cells that upregulate the expression of PROM-1/CD133 as a response to oncogenic mutations.

11.4 Stem Cells in Brain Tumors and Populations of Heterogeneous Tumor Cells

Two models have been proposed for explaining tumor initiation, cell heterogeneity and the nature of drug-resistant brain tumor cells. The conventional view of cerebral tumorigenesis is summarized in a stochastic model, which predicts that every cell inside the tumor is malignant and capable of initiating and maintaining growth because of the genesis of neoplastic (malignant) clones that also contribute to recurrence following a therapeutic intervention.

Tumor cells may have different potentials for proliferation, and they may propagate themselves with a stochastic probability. This heterogeneity has been attributed to genomic instability introduced by the initial oncogenic mutation, and by the selection looking for cells that may be better adapted to the tumor microenvironment. Typically, tumors that recur after an initial response to chemotherapy are resistant to multiple medications (multidrug-resistant). In the conventional view of tumorigenesis, one or many cells within a tumor acquire genetic changes that confer drug resistance. These cells have a selective advantage, which allows them to overcome the population of tumor cells with few mutations.

The second model is a hierarchic one for tumorigenesis, or a model for cancer stem cells. The proposed hypothesis is that a defined cell subtype, in this case cancer stem cells, possesses the exclusive ability to initiate and maintain their neoplastic (malignant) growth, and also for generating recurrent tumors. The pool of cancer stem cells grows in a series of hierarchic cell divisions generating phenotypically heterogeneous cells, similar to normal brain cells. Prototypic stem cells should maintain themselves by a relatively slow division in self-renewal processes, but they simultaneously give rise to cells resembling non-tumorigenic, highly proliferative and phenotypically diverse progenitor cells, and with a limited proliferative potential [18].

It is feasible that genomic instability within the population of cancer stem cells may lead to the accumulation of additional mutations that may also increase tumor heterogeneity. In this model, cancer stem cells are more resistant to therapies directed towards highly proliferative cell pools, such as radiation and cytotoxic drugs, and they are able to survive to conventional therapies in order to restore tumor growth [19]. Furthermore, brain tumor stem cells, as well as non-neoplastic (non-malignant) neural stem cells, would be able to express high levels of ABC transporters which may confer their multidrug-resistance ability [18].

11.5 The Cancer Stem Cell Hypothesis

Two experimental observations served for justifying the cancer stem cell hypothesis. First, large amounts of cells (>200,000), directly collected from the primary tumor tissue or derived from cell lines established by routine adherence, as well as serum-supplemented culture techniques are required for producing xenografts in immunosuppressed mice. This biological behavior contradicts the traditional stochastic model [20]. Secondly, cell lines cultured in serum and derived from high-grade human gliomas, do not represent every phenotypic feature and the large amount of genetic aberrations present in the corresponding primary human tumor [21]. One explanation for this may be that the conditions of the serum-containing culture used for establishing cell lines, do not select for tumor initiating cells, or they may non-reversibly alter their malignant potential. Alternatively, only subpopulations of tumor cells may have enough proliferative capability for generating tumor xenografts, and culture conditions with the presence of serum may not select for these malignant subpopulations. An additional support for the hierarchic model for tumorigenesis is phenotypic heterogeneity and the distinctive proliferative ability of neoplastic (malignant) cells, resembling that of normal neural stem cells.

11.6 Neural Stem Cells in the Mature Prosencephalon

The largest germinative region in the adult human brain is the subventricular zone in the lateral wall of the lateral ventricles. Few studies have carefully described this area [22, 23]. The germinal subventricular area is unique in adult mice because of a conspicuous hypocellular difference, and because of being arranged in more distinctive layers compared to those populated by different cellular phenotypes. A subpopulation of these astrocytic cells in the subventricular zone has proliferative potential and it also has the ability for forming neurospheres in cultures containing multipotential cells with self-renewal ability, and these may correspond to B1 cells in the murine subventricular zone. These cells with ultrastructural features similar to those of oligodendrocytes and displaced ependymal cells are also present in this layer, but with a smaller frequency. These oligodendrocytes do not seem to be

myelinated and the ependymal cell clusters do not have a definite orientation towards the ventricle. The IV layer contains myelinated appendages and it is the transition area between the subventricular astrocytes and the adjacent brain parenchyma. Radiate astrocytes may be differentiated in different phenotypes, including those cells from the subventricular zone in the III layer of the adult subventricular zone and parenchymal astrocytes [24, 25]. Proliferation levels in the adult subventricular zone are small compared to those found in other mammal species, while the cell architecture is quite different. Magnetic resonance imaging studies have revealed that glioblastomas associated with the subventricular area are multifocal, and they may recur at distant sites from the primary tumor [26]. This data indirectly points out towards a stem cell type or progenitor cell as the tumor initiating cell for a glioblastoma subgroup with a poor prognosis. The adult human subventricular zone also contains adult neural proliferating stem cells [25]. A possible scenario is that multifocal glioblastomas originated in mutated stem cells giving rise to mutated progenitor cells which migrate far away from germinal areas and proliferate in an aberrant form once they reach a favorable microenvironment. A more comprehensive analysis of adult neural stem cells and their progeny is necessary in order to determine whether they may give rise to glioblastomas because of the generation of neoplastic (malignant) stem cells.

Adult neural stem cells renew themselves in order to generate additional stem cells; and, depending on the temporal and positional information, they may give rise to neurons and glial cells. Neurogenesis also persists in the subgranular layer of the dentate gyrus in the hippocampus, which has a cellular hierarchy somehow similar to that of the subventricular zone [27].

Multipotential stem cells are slow when undergoing division and self-renewal, and they may be stimulated in order to give rise to rapidly-dividing cells, called transient amplifying type cells. The latter produce neuroblasts and glial progenitor cells [28, 29].

Multipotential stem cells have a prolonged life span and a marked proliferating capability, which makes them potential targets for the occurrence of an initial transforming mutation. Their similitude with glioma stem cells suggest that the population of malignant cells may be originated from the transformation of neural stem cells [5, 30, 31]. Neural progenitor cells positive for the intermediate filament nestin seem to be more sensitive to the transforming effect of the platelet-derived growth factor B (PDGF-B), for the epidermal growth factor receptor (EGFR), and to the combined activity of both Ras and Akt, respectively, compared to GFAP-expressing astrocytes [9, 32]. This data suggests that cell progenitors and maybe multipotential stem cells, rather than mature astrocytes, are the cellular origin of astrocytomas. The interpretation of these mechanisms is quite complicated by the fact that GFAP is also expressed in multipotential stem cells and not only in differentiated astrocytes [33].

The evidence of an immature cell as the source of neoplastic (malignant) stem cells has been corroborated by most recent data in which it was reported that an infusion of PDGF in the lateral ventricle of adult mice carrying the triple mutation was enough to induce type B cell proliferation, resulting in large hyperplasia

resembling glioma formation [29]. In one study, three tumor suppressing genes were deleted, namely neurofibromin-1, PTEN, and p53, specifically in neural stem/progenitor cells, nestin-positive, by inducible recombination of specific sites [34]. Cells that were nestin-positive in the subventricular zone in mice, developed premalignant (pre-neoplastic) defects, including advantageous features in both growth and maturation, and they developed into astrocytomas with complete penetrance. This data shows that immature cells, such as progenitor or stem cells are targets for mutations, and they give rise to astrocytomas.

11.7 Brain Tumor Stem Cells and Infiltrative Gliomas

11.7.1 Oligodendroglioma

The molecular pathogenesis of this tumors is not clearly understood, and it has been debated whether the cellular origin of oligodendrogliomas is a multipotential stem cell, a glial progenitor, or a differentiated glial cell. PDGFR and EGFR signaling are normally activated in normal oligodendrogenesis and in oligodendrogliomas, respectively [35]. PDGFR induces astrocyte dedifferentiation, which supports the notion that mature glial cells are the cellular origin of oligodendrogliomas. However, the similitude between these two oligodendrogenesis regulating pathways, the proliferation of oligodendrocyte and oligodendroglioma progenitors suggest that tumors arise from glial progenitors with a restricted lineage [36]. For example, ectopic EGFR stimulates the proliferation and inhibits the differentiation of oligodendrocyte progenitors; and, consequently, the oligodendrocyte progenitor type cells generate hyperplasia in the white substance [37]. Also, PDGFRα-positive neural stem cells generate oligodendrocytes in vivo. When PDGF was infused in adult mice cerebral ventricles, it induced massive proliferation of stem cells in the subventricular zone and large hyperplasia, similar to glioma formation with the expression of astrocyte markers, but not those of oligodendrocytes [29]. However, oligodendrogliomas in human and murine models predominantly express markers of mature oligodendrocytes or oligodendrocyte progenitor cells, such as NG2, PDGFRα and OLIG2, but no neural or astrocyte markers [38]. These observations suggest that restricted glial progenitors may progress to a malignant (neoplastic) state as a response to signaling of the ectopic growth factor receptors and the loss of tumor suppressors. Mutated restricted glial progenitors propagate the tumor by the generation of an excess of oligodendroglial progenitor type cells, at the expense of mature cells.

A small study identified CD133(+) neurospheres from a high grade oligodendroglioma [39]. The question remains, whether the expression of restricted glial progenitors such as NG2, rather than multipotential stem cells, might be the cellular origin of CD133(+) cells, which may be directed in murine models and not in human subjects. A model of oligodendrogliomas in transgenic mice expressing the verbB oncogene in restricted glial progenitors and lacking p53 developed

oligodendroglial tumors [12]. In this model, the verbB oncogene ectopically activates EGFR signaling in S100β(+) cells in the subventricular area and in the white matter throughout the brain to an early postnatal stage. Ectopic EGFR also induces pre-malignant changes, such as aberrant self renewal, altered differentiation throughout glial cell lines, and hyperproliferation in verb(+) neurospheres. More severe but similar changes were detected in stem cells from gliomas isolated from oligodendroglial tumors of S100-verbB p53 KO mice, based on their ability to form neurospheres.

Tumorspheres from gliomas fulfill criteria for defining them as brain tumor stem cells, including the expression of stem cell markers, aberrant self-renewal and in vitro differentiation, and also because of their ability to generate massive high grade oligodendroglial tumors [40]. Orthotopic tumors derived from tumorspheres imitate the features of high degree oligodendroglial type tumors [9], such as high celullarity, high mitosis index, perineural satellitosis, the typical 'fried egg' appearance of such cells and microvascular hyperplasia. Premalignant changes, such as increased self renewal, altered differentiation and hyperproliferation, as well as malignant progression, are accompanied by a change from asymmetrical cell division to a symmetrical cell division mode. This opens the possibility that an asymmetric cell division may prevent malignant changes and neoplastic progression. Glial progenitors in mammals with defects in asymmetric cell division may be genetically unstable, and then they might be predisposed to acquire additional mutations and experience neoplastic (malignant) transformation. As a support to our hypothesis, many confirmed oncogenes and putative tumor suppressors are well known regulators on asymmetric cell division [41]. The potential relationship between defects in asymmetric cell division and the development of gliomas should be investigated in greater detail.

11.7.2 Gliomatosis Cerebri

Gliomatosis cerebri is defined as an unusual glial neoplastic condition, which is biologically aggressive, with the presence of a characteristic marker of extension and dissemination of tumor cells in at least three brain lobes, which conspicuously preserve the underlying brain cytoarchitecture, including neuronal bodies and axonal structures. The invasive pattern may imitate subpial dissemination, neuronal satellitosis, perivascular location in the margins of infiltrative oligodendroglioma and glioblastoma tumors (secondary structures of Scherer), or a more amorphic diffuse dispersion pattern resembling low grade astrocytomas. In spite of the extensive involvement by tumor cells, there are no detectable masses using high resolution neuroimaging techniques. The presence of infiltrative tumoral cells is typically associated with a global volume increase, with a variable mass effect, of affected brain regions, showing minimally hypointense and isointense changes in T2 MRI (magnetic resonance imaging), and hyperintense changes using the FLAIR mode. Most commonly affected regions are the cerebral hemispheres, followed by the

mesencephalus, thalamus and basal ganglia, and, to a lesser extent, both the cerebellum and the brain stem. The hypothalamus, optic nerves and chiasm, as well as the spinal cord, seem to be affected in less than 10 % or reported cases. Even though the age range for the occurrence of these tumors is from the neonatal age up to the ninth decade of life, the mean age at the time of diagnosis is 12 years, and the peak incidence occurs between the fourth and the fifth decades of life in adults [42].

Phenotypic features of glial cell tumors are typically being astrocytic, but a small number of cases include cells with some oligodendroglial features, or mixed glial phenotypes [43, 44].

Tumor cells typically appear as small glial cells with a fusiform elongated nucleus with variable pleomorphism and hyperchromasia. Both necrosis and microvascular hyperplasia are always absent, which is consistent with the morphometric features that are most usually found in low degree gliomas. A quantitative study of vessels which showed a normal immunohistochemistry profile for microvasculature in brain regions affected by gliomatosis also confirmed that angiogenesis is completely absent in such lesions [45]. Mitotic indexes are quite variable (MIB $\leq$ 1–30), but they are typically low. However, some cases may occur presenting with greater cellular anaplasia and gliomatosis cerebri may progress in time to a higher degree phenotype [46].

Immunoreactivity for S100, GFAP, and MAP2 is present, but it is variable in most cases, similar to what has been found in low grade infiltrating gliomas [42, 47]. Even though a definitive molecular analysis of gliomatosis cerebri is troublesome because of the diffuse dispersion and low density of tumor cells in small biopsy samples, these neoplastic (malignant) cells seem to be clonal and they have molecular lesions similar to those found in low degree diffusely infiltrative gliomas [48]. Neoplastic (malignant) cells in gliomatosis cerebri express markers that are associated with motility in all degree infiltrative gliomas, CD44 (receptors for hyaluronic acid) and matrix metallopeptidases [49, 50]. However, two studies have pointed out key differences between low grade infiltrative gliomas and gliomatosis cerebri. A study of gliomatosis cerebri performed in a 29 year old male subject showed a predominant expression of FGFR1 mRNA (type β) (messenger ribonucleic acid) in biopsy specimens showing tumor cells with a typical low degree appearance [51]. FGFR1 expression most commonly occurs in malignant gliomas. This aberrant expression in gliomatosis cerebri may reflect a high proportion of migratory neural stem cell/early progenitor cells with an aberrant proliferative phenotype. During fetal development, the translocation of radial glia from the midline and the formation of the corpus callosum require FGFR1 signaling and this increases proliferation and inhibits spontaneous differentiation of adult neural stem cells by MAPK and Erk1/2 activation [52, 53]. The high immunoreactivity to nestin in GFAP(−) tumor cells in gliomatosis cerebri is also consistent with the hypothesis that proposes their origin from an early migrating stem/progenitor cell [54]. A recent study including four cases of gliomatosis cerebri showed an increased but variable expression of biomarkers related to Sox1 and Mushahi-1 stem cells. In contrast to glioblastoma, there was no significant CD133 expression in these cell populations [55].

11.7.3 Glioblastoma

Additionally to its similitude with neural stem cells, tumorspheres show specific tumoral capabilities, such as an increased self renewal rate, aberrant proliferation and differentiation, altered karyotype and expression of cell outcome markers; and most importantly, malignant features. Stem cells of brain tumors derived from murine and human tumorspheres faithfully reproduce the primary tumor from which they were derived after xenotransplantation [9, 21, 40]. The injection of tumorspheres from human glioblastoma generated tumors with typical features of high grade gliomas, such as slow growth, the presence of underlying necrotic areas with a typical pseudo-palisade appearance, an increase in microvascular proliferation and a high degree of mitosis. The most surprising finding is that implanted neurospheres are highly capable of migrating and they infiltrate the brain parenchyma with greater effectiveness compared to cell lines grown in serum, which is distinctive for high grade gliomas. Similarly to xenograft data, the results for large scale expression combined with a karyotypic analysis have shown that serum-free conditions in tumor sphere cultures preserve the overall expression profile and the phenotypic characteristics of original cells in a stronger way compared to serum containing regimens that have been traditionally used for establishing glioma cell lines [9, 21].

In an important study, Dirks Laboratory observed that CD133(+) cells in adult glioblastomas exhibited neoplastic (malignant) stem cell characteristics, while CD133(−) cells did not. Very few (100–1,000) CD133(+) cells were enough to induce the formation of a tumor after performing xenografts, and they are capable of being serially transplanted, while a greater number (100,000) of CD133(−) cells were not capable of undergo the same process. Glioblastoma xenografts obtained from CD133(+) cells consist in a smaller population of CD133(+) cells, and it is mostly comprised of CD133(−) cells, suggesting that there is a 'tumor hierarchy' in which tumoral CD133(+) stem cells proliferate in order to generate CD133(−) cells that are not stem cells [5, 30]. Although most human primary glioblastomas and tumorspheres variably express CD133 (20–60 %) [9, 39], some tumors may have very low fractions (<1 %), according to what has been determined by immunohistochemistry and flow cytometry studies. This may also be affected by the heterogeneous division speed of progenitor cell populations that may also be present. It is worth mentioning that one type of primary glioblastoma gives rise to CD133(−) cell clones with features similar to those of stem cells and somehow limited tumorigenicity, leading to the formation of slow growing and less infiltrative proliferative tumors [56]. An important question for future investigators is whether the differential status of CD133(+) cells and the different capabilities for tumor sphere formation in individual gliomas may reflect merely experimental differences or in fact these may be related to a different cellular origin for tumorigenesis. Since gliomas are initiated by many mutations and they may have multiple genetic defects, we anticipate that the markers of brain tumor stem cells may vary between glioma patients and they may reflect the heterogeneous nature of the tumor initiating

mutation and the cellular outcomes of individual tumors. A great challenge for personalized medicine will be the definition of specific features of brain tumor stem cells in individual patients with cerebral cancers. It is envisioned that the positive and negative selection for different cell surface markers, as well as that for specific intracellular signaling pathways and metabolic states may be used in the future for regularly isolating brain tumor stem cells from patients' tissue specimens.

11.8 A Critical View of the Stem Cell Hypothesis in Cancer

In spite of the recent advances in the study of tumor initiating cells in human gliomas, we are just beginning to understand their nature. A fundamental question requiring to be examined is whether stem cells from brain tumors in tumorspheres and tested in xenografts are in fact the tumor initiating cells in patients.

Lineage follow-up experiments aiming to determine the outcome of mutated cells, together with a better detection of brain tumor stem cells will guide the relationship between tumor initiating cells and tumor stem cells in murine models.

An important question remains unanswered, regarding the therapy assessment, whether similarly to multipotential stem cells in the subventricular area, the brain tumor stem cells may have a slow division ability, or they may be more similar to the so-called 'transient amplification' cells that show greater proliferation or behave as bipotential progenitors. Tumor sphere cells proliferate with a higher rate compared to normal neurospheres, and they normally grow independently of the presence of growth factors. Tumorspheres similar to neurospheres are heterogeneous, and they also contain progenitor cells. It is not known if tumor stem cells or progenitor cells may contribute to an increased proliferation of tumorspheres in vitro. Brain tumor stem cells in vivo are more similar to transient amplification cells and restricted lineage progenitor cells, which frequently proliferate, giving rise to different cell lineages.

Adult neural stem cell in the subventricular area form tight junctions with endothelial cells and they live in a vascular bed [57, 58]. A key question in the investigation of brain tumors is with respect to the role of microvascular tumoral stroma in affecting an analogous microenvironmental niche that may be able to regulate proliferation and self renewal of neoplastic (malignant) stem cells. Recent data also suggests that presumed tumor stem cells in medulloblastoma interact with endothelial cells from the tumor micro-perivascular niche [59]. This will be important in order to incorporate the effects of bidirectional signals between the microenvironment and tumor stem cells in any modeled system in order to elucidate the mechanism for tumor initiation and its maintenance.

A fundamental question in cancer biology is the extent of tumorigenic cells within individual tumors. Based on previous research performed in leukemia, the hierarchic model for tumorigenesis has initially suggested that tumoral stem cells are rare. However, the current research in neoplastic (malignant) stem cells in melanoma shows that the conditions of xenografting clearly determine the detectable

frequency of tumor initiating cells [60]. It has been proposed that a single stem cell may give rise to a single neurosphere, and that the number of neurospheres in one culture approximately corresponds to the number of stem cells within such culture [61]. However, the one-to-one relationship between stem cells and neuro/tumorspheres is difficult to prove from an experimental standpoint, and non-stem cells, such as transient amplification cells may form spheres in vitro [62]. Thus, it is likely that tumor sphere analyses underestimate the number of stem cells inside the tumor. It is necessary to adapt standardized isolation techniques, tumorspheres and the conditions of xenografting analyses in order to correctly estimate the number of brain tumor stem cells and compare these findings in patients with brain tumors in order to accurately determine the prognosis and the predictive value of stem cells in brain cancers.

Because of the similarities, it has been suggested that brain tumor stem cells arise from an adult stem cell or from immature progenitor cells rather than from differentiated cells. Recent data from murine models has been discussed, and it suggests that astrocytomas, oligodendroglioma and medulloblastoma arise from stem cells and/or progenitor cells. However, nowadays we do not fully understand the underlying mechanisms by which stem cells and progenitor cells progress to a neoplastic (malignant) state as a response to oncogenic mutations.

11.9 Therapy Implications of Tumor Stem Cells in Gliomata

Some small-sized studies have suggested that CD133 expression and the ability for neurosphere formation have prognostic value and that glioblastomas with CD133(−) cells and CD133(+) stem cells have different patterns of genic expression. In a large scale expression study, human glioblastomas were grouped in proliferative, proneural, and mesenchymal tumors. Neural stem cell markers, including CD133 and the formation of neurospheres were upregulated in molecular proliferative subtypes that correlate with a poor prognosis [63]. Thus, CD133 expression and the formation of tumorspheres are completely absent in secondary glioblastomas, which are histologically similar, but different from a molecular point of view with respect to primary glioblastomas [64].

Anaplastic oligodendrogliomas, oligoastrocytomas and glioblastomas with an oligodendroglial component are high grade oligodendroglial tumors, which are difficult to classify because of intratumoral diversity and the absence of clear cut histological markers. Since oligodendrogliomas and glioblastomas have different responses to therapy, an appropriate diagnosis is essential for obtaining good results. A small sized study correlated the frequency of tumor sphere growth and a CD133(+) population in high grade oligodendroglial tumors with a poor prognosis [39]. Taken together, the presence of CD133(+) stem cells or cell populations with other stem cell biomarkers, and the frequency of tumor sphere formation may become a useful criterion for predicting the response to therapy and for establishing new prognosis glioma subtypes. It is necessary to perform large scale studies in order to determine the prognostic and predictive value of CD133(+) stem cells and of cell populations

with other stem cell biomarkers, which may vary according to the glioma subtype. One study observed that brain tumor stem cells are more resistant to conventional therapy compared with non-stem cell tumor cells [65]. Nonetheless, more evidence is needed in order to conclude that glioma stem cells may survive to radiation and even to chemotherapy, and that they may give rise to recurrent tumors. The successful elimination of brain tumor stem cells with new stem cell targeted therapies may become as important as cytotoxic therapy targeting non stem cell neoplastic (malignant) cells in order to prevent tumor growth and recurrence.

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Chapter 12
Sarcomagenesis

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Abstract Sarcomas represent a heterogeneous group of neoplasms arising from the malignant transformation of mesenchymal cells. Evidence has increased considerably regarding the origin of sarcomas having putative sarcoma stem cells which are responsible for the initiation, maintenance, differentiation and proliferation of osteosarcoma, synovial sarcoma, rhabdomyosarcoma and Ewing's sarcoma. Different methods have been adopted for identifying primitive cells in sarcomas such as identifying surface markers, using flow cytometry for isolating cells having aldehyde dehydrogenase activity and performing side population analysis. This chapter summarizes and discusses data regarding the tumorigenesis of sarcomas, assessing their potential role in sensitivity and resistance to different classical interventions (chemotherapy and radiotherapy) as well as new molecularly-directed therapies.

Keywords Sarcoma • Stem cell • Mesenchymal cell • Genotype

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12.1 Introduction

Sarcomas represent a heterogeneous and uncommon group of malignancies, arising from connective tissues whose primary function is to support an organism and its systemic integration. Together, they account for over 20 % of all pediatric solid malignant tumors but less than 1 % of all adult malignancies. The vast majority of diagnosed sarcomas arise from soft tissues, while malignant bone tumors make up just over 10 % of all sarcomas [1]. Sarcomas affect ~11,000 individuals annually in the USA and around 200,000 worldwide [2, 3]. Risks for sarcomas developing can be divided into environmental exposure, genetic susceptibility, and an interaction between them. Radiotherapy has been strongly associated with secondary sarcoma occurrence as the history of hernias has revealed a greater risk of Ewing's sarcoma (EWS) developing among children [4, 5]. Bone development during pubertal growth spurts has been associated with the development of osteosarcoma and exposure to chemicals such as herbicides whilst chlorophenols have also been linked to how sarcomas originate [1].

Sarcomas have been historically grouped into two main types according to tumor location: soft tissue sarcoma (STS) and primary bone sarcomas; however, an alternative genetically-based classification has divided sarcomas into two broad categories since 2002 [6], each including clinically-diverse tumor subtypes. The first includes sarcomas having near-diploid karyotypes and simple genetic alterations, including translocations or specific activating mutations (alveolar rhabdomyosarcoma, myxoid liposarcoma, EWS and synovial sarcoma); the second covers tumors having complex and unbalanced karyotypes characterized by genome instability resulting in multiple genomic aberrations (leiomyosarcoma, malignant fibrous histiocytoma and osteosarcoma) [7, 8]. Such genomic subtypes seem to be related to a common subpopulation of self-renewing cells capable of initiating sarcomas and maintaining them in the long-term. Increasing evidence has suggested that multi-potent mesenchymal stem cells (MMSC) reproduce human sarcomas upon the overexpression of specific fusion oncoproteins or disruption of key signaling pathways [9]. Ex vivo MMSC have certain dominant characteristics including adhesion plasticity.

CD105, CD73 and CD90 expression and lack of reactivity to CD45, CD34, CD14, CD11b, CD79b, CD19 and HLA-DR occur when MMSC are kept in standard culture conditions. Likewise, MMSC should be capable of differentiating into osteoblasts, chondroblasts and fat cells in vitro [10]. The exact nature and localization of MMSC in vivo remain poorly understood, but recent data has indicated that sarcoma precursors could have a perivascular distribution [11, 12], their niche would include several cell subsets spanning different stages of mesodermal development having distinct potency, ranging from multi-lineage stem cells to unilineage precursors or even fully-differentiated cells [13]. The expression of embryo markers, such as Oct-4, in tumor and aged MMSC represents another finding supporting the idea of sarcomas having a common origin [14].

The present chapter has been aimed at presenting and discussing evidence related to the origin of sarcomas, following the hierarchical principle of a primordial cell model.

12.2 The Genetic Taxonomy of Sarcomas

Most sarcomas involving simple genetic alterations have translocations and account for around a third of such neoplasms; they tend to be presented *de novo* and some of the cytogenetic damage so caused is retained through clonal evolution. Most fusion genes encode chimeric transcription factors causing transcription alterations, whilst others encode proteins having tyrosine kinase or growth factor activity [15].

By contrast with sarcomas derived from well-recognized translocations, the second group involves complex karyotype modifications arising from less aggressive forms and runs through different stages of the disease, each having greater complexity. Liposarcoma, peripheral nerve-derived tumors and chondrosarcomas are clear examples of such subgroup. The main mechanisms triggering sarcomagenesis are associated with transcriptional deregulation producing aberrant fusion proteins arising from genomic rearrangements as well as the presentation of somatic mutations in driver genes from differing signaling routes and abnormalities regarding the number of DNA copies. The importance of telomere maintenance-associated genome integrity has also been recognized. Major telomerase activation in the absence of alternative lengthening of telomeres (ALT) characterizes sarcomas having specific chromosome translocations; nevertheless, ALT occurs more frequently in sarcomas having non-specific complex karyotypes [16, 17]. Lafferty-Whyte et al., have described a genetic signature which led to classifying telomerase and changes in ALT for pluripotent cell mesenchymal transition [18].

Sarcomas having non-specific complex karyotypes are sometimes found which have no association with the translocations regularly present in hereditary syndromes produced by genomic instability, such as the Werner (WRN), Nijmegen Breakage (NBS1) and Rothmund-Thomson (RECQL4) syndromes [19–21].

Studies of the genome's complete sequence have found that around 35 % of osteosarcomas and 18 % of chordomas have chromothripsis; this involves hundreds of chromosome rearrangements occurring during a particular cell crisis. Such catastrophe has been described in up to 3 % of neoplasms but appears in a quarter of high-grade bone tumors and in medulloblastoma of children predisposed by germinal mutations in p53 [22–24]. The most representative examples of transcriptional regulation amongst sarcomas are associated with the PAX3-FOXO1 fusion protein whose direct objective would include myogenic genes such as myogenic differentiation 1 (MYOD1) and myogenic factor 5 (MYF5), as well as other biologically-active elements such as fibroblast growth factor receptor 4 (FGFR4), anaplastic lymphoma kinase (ALK), mesenchymal epithelial transition growth factor (c-MET), insulin like growth factor 1 receptor (IGF1R) and neuroblastoma-derived, myelocytomatosis viral related oncogene (MYCN) [25, 26].

The ASPSCR1 gene becomes fused to transcription factor TFE3 (IGHM enhancer 3) in alveolar sarcoma to form a chimeric protein retaining the TFE3 DNA binding domain (containing the CACGTG recognition site). Recognition studies have found that such alteration is related to the activation of MET uridine phosphorylase 1 (UPP1) and CYP17A1 genes (cytochrome P450 17A1) [27].

A somewhat more complicated picture has emerged concerning EWS which affects Ewing sarcoma breakpoint region 1 (EWSR1) and Friend leukemia virus integration 1 (FLI1) genes [28]. Several ChIP-seq datasets have been produced in EWS cell lines with endogenous EWS-FLI1, all using the same FLI1 antibody for immunoprecipitation of EWS-FLI1-bound DNA. The amount of bound genomic regions in such studies has varied widely [14–16]. ChIP-seq has demonstrated that most EWS-FLI1-bound genomic regions were intergenic and that EWS-FLI1 bound avidly to GGAA microsatellites through its FLI1-derived ETS family DNA-binding domain [28, 29]. Microsatellites containing 6 or more GGAA repeats (the core ETS domain binding sequence) have been associated with EWS-FLI1 target gene upregulation [28, 30]. These repeats are often more than 200 kb upstream of the target gene transcription start site, suggesting that chromatin looping brings distant regions together in a transcriptional hub to allow EWS-FLI1 to modulate gene expression. EWS-FLI1 also binds to more conventional, non-repetitive ETS motifs and such sites are associated with genes repressing or activating transcription [30]. A subset of EWS-FLI1 target regions has shown co-enrichment of sites for E2F, nuclear respiratory factor 1 (NRF1), and nuclear transcription factor Y (NFY), thereby raising the possibility of specific cooperative interactions [31].

On the other hand, some EWS cell lines may be able to reprogram themselves, as such events have been documented after the EWS-FLI1 gene has been silenced, thereby producing a more similar expression profile to that of mesenchymal stem cells (MSC) which might then be induced to become differentiated by adipogenic or osteoblast linage [32, 33]. For example, EWS-FLI1 has induced limited expression of a neuroectodermal gene which can program and impose an osteogenic differentiation mold by inhibiting Runt-related transcription factor 2 (RUNX2) which is related to other genes promoting bone maturation. EWS-FLI1 expression in MSC has induced EWS in a reverse experiment; on the contrary, EWS-FLI1 expression has provoked apoptosis in other differentiated cells presenting intact ARF-p53 [34].

EWS-FLI1 directly upregulates the polycomb group repressor enhancer of zeste homolog 2 (EZH2) in human MSC, [35] and has induced expression of embryonic stem cell genes POU5F1 (also known as OCT4), SRY-box 2 (SOX2) and NANOG, at least partly by repressing miR-145 expression [36]. Interestingly, EWSR1 also fuses with POU5F1 itself, albeit rarely, in undifferentiated bone sarcoma [37, 38], myoepithelial tumors of the soft tissue [39], and in certain salivary gland tumors [40].

Synovial sarcomas contain fusions between the SS18 (SYT) SSX1 or SSX2 genes. Analogously to that found in EWS-FLI1, synovial sarcoma cell lines also express POU5F1, SOX2 and NANOG. Silencing SYT-SSX fusion in such cell lines has increased their differentiation potential regarding adipogenic, osteoblast or chondrogenic linages [41]. Synovial sarcoma formation in mice accompanied by the conditional expression of SYT-SSX2 in myoblasts or in other cell linages has provided additional information about fusion protein nuclear reprograming in a compromised variety of mesenchymal linages. Some myxoid liposarcoma fusions, such as FUS-DDIT3 (SHOP) and ARMNS (PAX3-FOXO1), seem to have been able to transform mesenchymal progenitors in murine models. Figure 12.1 includes genotype lineage between sarcoma subtypes.

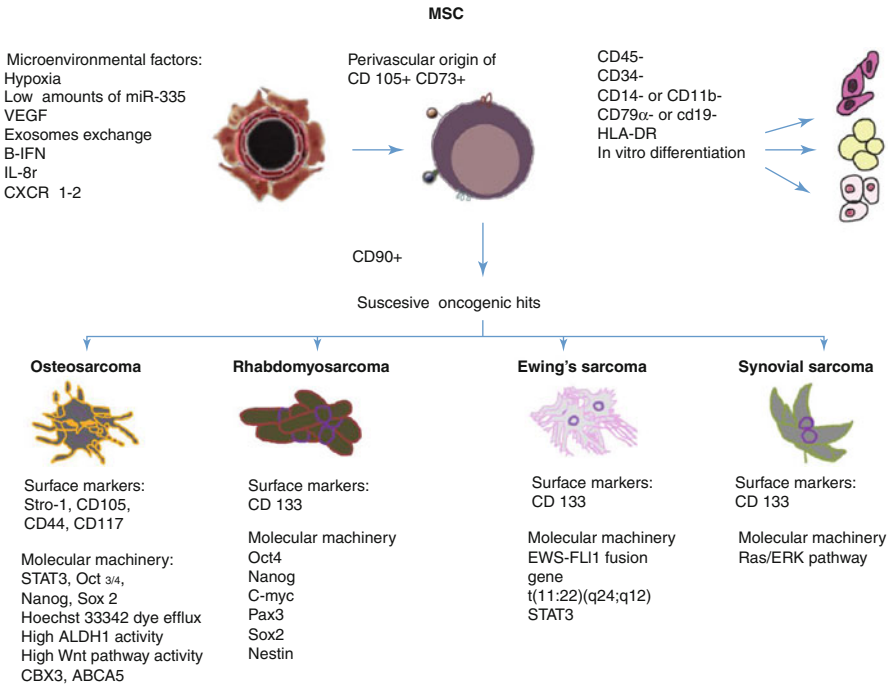

Fig. 12.1 Genotype lineage between sarcoma subtypes

12.3 Mutations and Signaling Routes in Sarcomas

Excluding gene fusions in sarcomas having translocations, it can be stated that few driver genes have recurrent mutations. The most representative examples would be angiosarcomas, an aggressive vascular tumor which has been shown to overexpress tyrosine quinase receptors in some transcription profiles, including KDR (VEGFR2), TIE1, SNF related kinase (SRNK), TEK and FMS-related tyrosine kinase 1 (FLT1) [42]. Sequencing these 5 genes has revealed that 10 % of angiosarcomas have mutations in KDR, and there has been independent ligand activation when mutant VEGFR2 proteins have expressed COS-7 cells. Large-scale genomic analysis of seven types of sarcoma has identified mutations in TP53, NF1 and PI3KCA [43]; 17 % of pleomorphic liposarcoma have mutations in TP53, such finding being consistent with the fact that such alterations are frequent in tumors having complex karyotypes. On the contrary, alterations in TP53 and homozygous deletions in cyclin-dependent kinase inhibitor 2A (CDKN2A) have been less common in translocation-associated sarcomas but, when present, have usually been related to a very aggressive clinical course [44]. Eighteen percent of myxoid/round cell liposarcomas have mutations in PI3KCA, thereby suggesting their role as modifications cooperating with the fusion protein (FUS-SHOP) in developing sarcomagenesis [45, 46]. Curiously, mutations found in PI3KCA have been located in the two

hotspots observed in epithelial tumors: the helical domain (E542K and E545K) and the kinase domain (H1047L and K1047R). Patients having mutations in the helicoid domain have a lower chance of survival attributable to the disease; they have increased AKT phosphorylation in CREB-regulated transcription coactivator 2 (TOR2) and in pyruvate dehydrogenase kinase 1 (PDK1) [45, 46].

Another recent finding has concerned precise NF1 mutations or deletions being present in 10 % of mixofibrosarcomas and 8 % of pleomorphic liposarcomas. This finding has been associated with individuals presenting neurofibromatosis type 1 (alterations in the germ-line and somatic mutations) but has not been described previously in subjects having sporadic tumors [43].

A special point deals with genomic alterations of gastrointestinal stromal tumors (GIST); mutations in KIT and, to a lesser extent, in PDGFRA are considered primary effectors of the disease, meaning that they are routinely identified in clinical practice before treatment is begun. Physiologically, these receptors are activated after ligand binding, thereby triggering receptor dimerization followed by autophosphorylation of the intracellular tyrosine kinase domain and final activation of multiple substrata included in the signaling pathway, such as PI3K/AKT, RAS, MAP and JAK/STAT. Mutations in KIT and PDGFRA are mutually exclusive in GIST and around 10 % of these tumors have a wild genotype; some recent series have described the presence of the BRAF gene V600E mutation in up to 7 % of these patients [45–47]. Until quite recently, no mutations had been detected in KRAS in GIST patients having alterations in KIT; however, Antonescu et al., have identified mutations in codon 12 (G12D: GGT->GaT), 13 (G13D: GGC->GaC), and a concomitant variation (G12A/G13D: GGT->GcT and GGC->GaC) in KRAS in three patients who had no record of prior exposure to imatinib (5 %) [48]. Another group of GIST patients (children) has overexpressed IGF1R MRNA and its protein, even though the mechanism for such alteration remains unknown. In fact, most pediatric tumors have diploid genomes [49].

12.4 Alterations in the Number of Gene Copies

DNA copy-number alterations provide the third route for sarcomagenesis. Sarcomas have a range of complexity among human malignancies regarding their copy-number alterations [50]. They vary from translocation-associated sarcomas with few copy-number alterations (broad or focal) to karyotypically-complex subtypes that are heterogeneous, unstable and profoundly altered regarding their genomic copy number. Moreover, recent high-resolution array-based copy-number analysis has revealed an intermediate complexity group characterized by few, yet highly recurrent, amplifications exemplified by undifferentiated liposarcomas [43]. Information from another copy-number analysis has shown that the third category can be subdivided into sarcomas having few chromosome arms or whole chromosome gains or losses and sarcoma genomes having a high level of chromosomal complexity [51].

Intermediate complexity sarcomas, such as well-differentiated and undifferentiated liposarcomas, are driven by chromosome 12 alterations, often generating extrachromosomal episomes, ring chromosomes and larger markers [52]. These 12q gains have high prevalence (80–90 %) and co-amplified oncogenes cyclin-dependent kinase 4 (*CDK4*) and *MDM2* can serve as confirmatory diagnostic markers [53] and as targets [54]. Another gene affected by 12q amplification is *HMGA2*, which often loses its 3' untranslated region (UTR), disrupting microRNA-mediated repression [55]. This genetic remodeling of chromosome 12 is likely the result of progressive rearrangement and amplification in an evolving amplicon rather than a single catastrophic event such as the recently-proposed chromothripsis. Similar 12q amplifications occur at lower frequencies in other mesenchymal tumors such as osteosarcomas [56]. Other remarkable, and less frequent, amplifications in the intermediate sarcoma group occur on 1p and 6q; such amplifications, which appear to be mutually-exclusive, span genes in the p38 and JNK pathways of MAPK signaling including, on 1p, *JUN* and, on 6q, *TAB2* and *MAP3K5* (*ASK1*) [57, 58]. Another genomic amplification alteration is the telomerase reverse transcriptase (*TERT*) gene located on 5p [43]. Some genomic amplification targets appear to be shared among a subset of both intermediate and highly complex sarcomas, including Yes-associated protein 1 (*YAP1*) and vestigial like 3 (*VGLL3*) on 11q22 and 3p12, respectively [59].

On the other hand, highly complex sarcomas harbor multiple numerical and structural chromosome aberrations that are similar to those previously described in epithelial tumors. Molecular classification of these subtypes reflects varying levels of similarity in their genomic aberrations; some subtypes may be considered a single entity [60], while others are distinct [61]. Broad amplification of several chromosome arms (such as 5p) [62] often occurs in combination with deletions affecting well-established tumor suppressors such as *CDKN2A*, *CDKN2B*, *PTEN*, retinoblastoma 1 (*RB1*), *NF1* and *TP53*. In fact, several of these genes play a direct role in maintaining chromosome integrity [63] and their loss of function may be an early event leading to genomic instability in highly complex sarcomas. Genomic deletions are more common than amplifications in other subtypes, such as leiomyosarcoma [63].

12.5 Genesis of Primary Sarcomas

It has been established recently that transformed MMSC may initiate sarcomagenesis in vivo. Efforts have been directed towards characterizing such transformation and also to prospectively generating specific models for different sarcomas. These studies include both spontaneous and induced MMSC transformation mediated by specific alterations such as an accumulation of chromosome instability, p53 mutations or loss of CDKN2A/p16. Mouse MMSC is especially predisposed to acquiring such alterations after long-term in vitro culture favoring clonal selection [64–67]. p53-depleted mouse adipose-derived MSC (mASC) have been capable of originating leiomyosarcoma-like tumors after injection into immunodeficient mice. This

finding has been supported by a differentiation-based microRNA study which identified leiomyosarcoma as an MSC-related malignancy [68, 69]. Another study determined that complete loss of p53 expression in p21−/−p53+/− mASC after culture induced cell growth, karyotype instability and loss of p16INK4A which prevents senescence, thereby resulting in the formation of fibrosarcoma-tumors in vivo [70]. Overexpression of c-MYC in p16INK4A−/−p19ARF−/− bone marrow mouse MMSC has resulted in osteosarcoma developing, accompanied by a loss of adipogenesis. Similarly, the loss of other cell cycle regulators, such as Rb, has not transformed mMSC but its deficiency has potentiated tumor development of p53-deficient mouse MMSC, generating further undifferentiated sarcomas [71].

Although Rb-deficient mice have developed normally, Rb deficiency has synergized with p53 deletion to accelerate sarcoma formation and increased the frequency of poorly-differentiated sarcomas.

In other mouse models where mutations have been restricted to muscle, the expression of oncogenic K-RAS or the mutation of endogenous K-RAS has been needed to efficiently induce sarcoma formation in p53-deficient tissue [72].

Sarcomas developed in these models have been characterized as pleomorphic rhabdomyosarcoma and high-grade sarcomas with myofibroblastic differentiation. Interestingly, deletion of the INK4A-ARF locus could substitute the p53 mutation in such K-RAS mutation-based model of sarcoma development [73].

Human MMSC do not undergo malignant transformation as easily as mouse primitive cells. For instance, as opposed to mouse MMSC, inactivation of p53 or p53 and Rb has not induced transformation in humans, although p53-/Rb-deficient human MMSC have displayed a higher in vitro growth rate coupled to an extended lifespan [74, 75].

Several oncogenic events must be combined to promote in vivo sarcomas from human MMSC, including introducing the human telomerase catalytic subunit (hTERT), HPV-16 E6 and E7 (abrogating p53 and Rb family member functions), SV40 small T- or large T-antigens (resulting in c-MYC stabilization and inactivating Rb and p53, respectively) and oncogenic H-RAS (providing a constitutive mitogenic signal) [76, 77].

In one striking model, transforming human MMSC has been associated with a gradual increase in genomic hypomethylation, although this is not necessary for sarcomagenesis. Using a different basic approach, another research group has transformed human MMSC through ectopic expression of hTERT, H-RAS and BMI-1, thereby inhibiting the expression of polycomb response element-controlled genes, including p16INK4A [78].

It has also been reported that some hTERT-transduced human MMSC lines have lost contact inhibition, acquired anchorage-independent growth and formed tumors in mice after long-term in vitro culture. This has been associated with the deletion of the Ink4a/ARF locus and with acquiring an activating mutation in K-RAS. Overall, in vivo tumors originating from most of these transformed human MMSC have been classified as undifferentiated spindle cell sarcomas [77].

Besides inactivation of cell cycle regulators, hMSC transformation has been related to alterations in several signaling pathways. It has been reported that the

PI3K-AKT-mTOR signaling pathway plays a critical role in the development of leiomyosarcomas.

Mice carrying a homozygous deletion of PTEN in the smooth muscle have thus developed leiomyosarcoma. PTEN and PI3KAKT involvement in leiomyosarcoma has been implicated by the fact that these signaling pathways have been dysregulated in leiomyosarcoma-forming p53-decifient mouse MMSC [79].

The WNT/β-catenin pathway plays a major role in the balance between self-renewal, differentiation, regulation and invasion of human MMSC. A loss of WNT characteristics in MMSC leads to malignant transformation and reduces apoptosis; accordingly, a recent study has supported a role for aberrant β-catenin stabilization in promoting MMSC-derived tumorigenesis [80]. Similarly, inactivation of WNT signaling upon treatment of previously SV40-immortalized human MMSC with the WNT inhibitor DKK1 has led to full malignant transformation of these cells and the consequent in vivo formation of malignant fibrous histocytoma [81].

Conversely, restoring WNT signaling in sarcoma cells has allowed them to differentiate amongst different mesenchymal lineages. It has been reported that key components of the WNT pathway are down-regulated in osteosarcoma compared to normal human MMSC and MMSC differentiated into osteoblasts [82].

12.6 Osteosarcoma

Osteosarcoma (OS) is the most frequently occurring primary bone sarcoma, accounting for around 20 % of all bone tumors and about 5 % of overall pediatric tumors [83]. OS is the fifth most common malignancy among individuals aged 15–19 years and the second most common in adolescence after lymphoma. OS has a bimodal age distribution, the first peak occurring during the second decade of life and a second peak in elderly adults [84, 85].

Higher incidence has been reported in boys and in African-American children. Areas having rapid bone growth are the most common locations in young adults, including the distal femur, proximal tibia, and proximal humerus. Nevertheless, OS is rare, less than 1,000 new cases being diagnosed per year in the USA, accounting for less than 2 % of all new cancer cases reported there [86].

Exposure to beryllium oxide [87], orthopedic prostheses [88], and the FBJ virus [88] has caused OS in animal models; however, their role in human OS remains unknown. SV40 viral DNA has been detected in up to 50 % of OS tumors [88] while it is unclear whether SV40 plays any role in OS tumorigenesis [89]. Radiation exposure is a well-documented risk factor for OS, but the interval between radiation exposure and tumor appearance is long and hence is likely to be irrelevant concerning the development of most conventional OS tumors. Nevertheless, radiation could be responsible for the development of secondary post-radiation therapy OS regarding certain primary tumors [84, 90]. Increasing evidence suggests that OS may be considered a differentiation disease [83, 84, 90].

Nearly 70 % of OS tumors display a multitude of cytogenetic abnormalities. The ploidy number in OS has ranged from haploidy to near-hexaploidy; 1p11–p13, 1q11–q12, 1q21–q22, 11p14–p15, 14p11–p13, 15p11–p13, 17p, and 19q13 chromosomal regions are most commonly involved in structural abnormalities.

On the other hand, the most frequently detected amplifications include chromosomal regions 6p12–p21 (28 %), 17p11.2 (32 %), and 12q13–q14 (8 %). Several other recurrent chromosomal losses (2q, 3p, 9, 10p, 12q, 13q, 14q, 15q, 16, 17p, and 18q) and chromosomal gains (Xp, Xq, 5q, 6p, 8q, 17p, and 20q) have also been identified, as well as several recurrent breakpoint clusters and non-recurrent reciprocal translocations.

Osteosarcoma stem cells express Stro-1, CD44, and CD105 MSC markers [83, 84] and preferentially express key marker genes for EWS cell pluripotency, including Oct3/4, Nanog, Stat3 and Sox2 [84–86]. Oct3/4 expression is believed to play a vital role in tumorigenesis; however, Oct3/4 expression studies on tumors are usually carried out without considering isoforms as the existence of two mRNA protein Oct3/4 isoforms (Oct3/4A and Oct3/4B) has been validated [87].

Wang et al., have examined these Oct3/4 isoforms in osteosarcoma; their study demonstrated that Oct3/4A expression was significantly up-regulated in OS99-1, Hu09 and MG63 cells compared to Saos-2 cells, suggesting that lower Oct3/4A expression may be seen in non-tumorigenic cells since Saos-2 is a non-tumorigenic cell line while others are tumorigenic cell lines. Oct3/4B expression in the Hu09 cell line was significantly higher than in the OS99-1, Saos-2 and MG63 cell lines. The higher Oct3/4B expression noted in the Hu09 cell line may have reflected its aggressiveness, since this human osteosarcoma cell line is known to have a high rate of metastasis in the lungs of nude mice after intravenous injection [88].

Osteosarcoma stem cells are driven by specific signaling pathways; Shh, Dhh, PTCH1, SMO, GLI1 and GLI2 transcripts have been over-expressed in the osteosarcoma cell line. Recent research has shown that the HH pathway has been activated in osteosarcomas and cyclopamine can prevent such tumor growth by cell cycle regulation [83, 84, 89, 90].

Research concerning the NOTCH pathway has found that γ-secretase complex inhibitors deplete stem cells and slow NOTCH-dependent tumor growth, thereby agreeing with a study which has shown that the NOTCH pathway is activated in osteosarcoma and that γ-secretase inhibitors hinder osteosarcoma growth by cell cycle regulation. The Wnt/β-catenin pathway is another focused pathway which is often inactive in conventional high-grade osteosarcomas. Interestingly, CD99 could inhibit osteosarcoma by acting through the Wnt/β-catenin pathway [83, 84, 90].

The MAPK pathway has also been observed to play an important role in osteosarcoma pathogenesis. ERK, JNK and p38 (MAPK pathway components) form an inter-coordinating network and regulate cell proliferation, differentiation, apoptosis, invasion and migration in osteosarcoma. Arsenic trioxide has been shown to inhibit osteosarcoma cell invasiveness via the MAPK signaling pathway. Other pathways linked to osteosarcoma stem cells include Fas/FasL and transcription 3 (Stat3) [84, 90, 91].

12.7 Ewing's Sarcoma

The Ewing sarcoma family of tumors, including Ewing's sarcoma, peripheral primitive neuroectodermal tumors and Askin tumour, have poorly-differentiated, small round blue cells which appear in bones and soft tissue. Together, they represent at least 10 % of sarcomas (1–3 cases per million people/year), usually occurring more frequently in Caucasian adolescents and young adults, presenting non-random chromosomal, balanced alterations in the EWS gene from chromosome 22 and in ETS, usually becoming fused with the FLI1 gene from chromosome 11 [92–94]. However, alterations have also been found due to inversion, insertion and translocation with ERG [95]. The t(11;22)(q24;12) translocation product generates a chimerical protein representing Ewing sarcoma pathogenesis; the EWS protein is an RNA binding element and FLI1 is a transcription factor binding to DNA to modular diverse genes responsible for controlling cell apoptosis and differentiation [96].

Tirode et al., have described the possible origin for Ewing's sarcoma as being MMSC originating from bone marrow or soft tissue [97]. They revealed a potential relationship between Ewing sarcoma and MMSC by evaluating the effect of silencing EWS-FLI1 on gene expression and Ewing sarcoma cells' biological properties. They produced expression data from Ewing sarcoma cells after silencing EWS-FLI1 and found that the gene expression profiles shifted toward those for two types of MMSC culture. The changes included increased expression of several genes often expressed in several MMSC cultures. A number of neural genes characteristically expressed in Ewing sarcoma were also down-regulated after EWS-FLI1 silencing. These results suggested that EWS-FLI1 did indeed alter Ewing sarcoma progenitor expression, leading to loss of markers which might be expressed in the original stem cell and aberrant expression of markers which are normally absent in progenitor cells. In addition to the aforementioned modifications in gene expression, Tirode et al., also observed changes in Ewing sarcoma cell phenotype after EWS-FLI1 expression had been silenced. Strikingly, like MMSC, the silenced cells could more readily be induced to differentiate along osteogenic or adipogenic lineages than control cells.

Luca Suva et al., isolated a CD133+ Ewing sarcoma cell subpopulation displaying the ability to initiate and sustain tumor growth through serial transplantation in non-obese diabetic/severe combined immunodeficiency mice [98]. Quantitative real-time PCR analysis of genes implicated in stem cell maintenance revealed that CD133+ Ewing sarcoma cells expressed significantly higher levels of OCT4 and NANOG than their CD133- counterparts, thereby confirming their primitive origin. Significantly higher levels of key EWS-FLI1-regulated genes which are required for the tumorigenic phenotype, such as NKX2.2 and NR0B1, were also expressed in patient-derived Ewing sarcomas [34].

EWS-FLI1 expression resulting in p53-dependent growth arrest is another point implicated in Ewing sarcoma origin in hTERT-immortalized human fibroblasts. This has suggested that EWS-FLI1 is toxic when expressed in an improper cellular context. Mutation in p53, or other p53 pathway components, may then allow for

stable EWS-FLI1 expression and such cells' growth and survival. Although mutations in p53 itself are present in only 10–15 % of Ewing sarcoma cases, other alterations may occur in the p53 pathway (loss of p14ARF/p16CDKN2A, or HDM2 amplification). p53 activity in Ewing sarcoma cells may also be modulated by EWS/FLI-mediated inhibition of Notch pathway signaling [99–102].

Another important finding has concerned abnormal EWS-FLI1 expression upregulating proteins such as the neuron-specific microtubule gene (MAPT), the parasympathetic marker cholecystokinin, and the epithelial marker keratin 18 which are all capable of inducing the Ewing sarcoma neural crest phenotype [103].

12.8 Conclusions

Sarcomas are usually studied when full transformation events have already occurred, meaning that transformation and pathogenesis mechanisms are not therefore amenable to analysis with patient samples. There is thus a need to establish bona fide mouse- and human-based models for recapitulating sarcomagenesis in vitro and in vivo; mounting evidence during recent years has indicated that MMSC from different sources may represent the putative target cell for a variety of human sarcomas, thereby linking MMSCs and cancer. Future research should be aimed at defining precisely the specific phenotype for MMSC populations at the origin of the different types of sarcomas as well as ascertaining the pertinent mechanisms governing MSC transformation. It is envisioned that MMSC-based experimental research taken together with whole-genome sequencing of different types of primary sarcomas will advance attempts to develop accurate MSC-based models of sarcomagenesis and decipher the underlying mechanisms. This would provide a better understanding of the onset and progression of mesenchymal cancer and lead to the eventual development of more specific therapies directed against sarcoma-initiating cells.

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Chapter 13
Cancer Stem Cells in Gynecologic Cancer

Juan Carlos Mellídez Barroso and Maria C. Santos

Abstract Gynecological cancer is currently the sixth leading cause of death among women. Despite screening campaigns, early diagnosis and vaccination against Human Papilloma Virus, gynecological cancers -mostly ovarian cancer- are often diagnosed at advanced stages. Although the high effectiveness of existing medical treatments, during long term therapy, often seem insufficient to achieve the cure or the disease. This is due to the presence of metastases at the time of diagnosis and to the development of resistance to cytostatic treatments and further recurrences.

The discovery and understanding of Cancer Stem Cells (CSCs) leads to an encouraging outlook on the future treatment of these patients. Within the perspective of the CSCs concept model, gynecological CSCs have specific genetic and epigenetic variations that give them capabilities to undergo asymmetric cell divisions, ensuring new generations of CSCs and of more differentiated gynecological cancer cells. As a result of these biological capabilities, CSCs can mediate gynecological cancers occurrence, resistance to treatment and recurrence.

In this review we discuss emerging evidences supporting the existence of CSCs in ovarian, endometrial and cervical cancers. Focus is given to recent molecular and genomic advances regarding the characterization of abnormal signaling cascades in these three types of gynecological CSCs. We also discuss the current knowledge on genetic and epigenetic changes in gynecological cancers and CSCs, and how researchers propose that these cellular changes influence CSCs in their control of cancer development and recurrence.

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We also discuss advances in medical treatment of gynecological cancer using CSCs as a therapeutic target.

Keywords Cancer stem cells • CSC • Gynecological cancers • Human Papilloma Virus • Ovarian cancer

13.1 Introduction

In 1858, the German pathologist Rudolf Virchow postulated that cancer develops from immature cells. Nowadays this hypothesis seems to be confirmed with the discovery, identification and isolation of the so-called Cancer Stem Cells (CSC). These cells have been identified in most cancer types and are considered responsible for several aspects of the behavior of malignant tumors.

However, the biological role of CSCs in carcinogenesis, invasive phenotype, resistance, metastasis, recurrence and cancer therapy is still a matter of intense study and discussion [144].

It is assumed that tumors originate from a single Tumor Initiating Cell (TIC) that originate, by asymmetric division, an identical daughter and another one that will differentiate. These differentiated daughter cells yield a mixture of heterogeneous populations of phenotypes that constitute the tumor mass [2]. The stromal microenvironment, nutrition niche and adequate oxygenation allow tumor development [63].

The initial biological event of cancer development occurs long before clinical manifestations of the tumor [131]. It is speculated that this event (including DNA damage, mutations, oncogene activation, epigenetic changes, etc.) occurs in a CSC, that, once mutated may remain quiescent for months or years, initiating tumor development when activated [2].

Similarly to the healthy Stem Cell, the CSCs have unlimited proliferative potential and an unlimited renewal capacity in an undifferentiated state [58, 101]. They also have the ability to divide asymmetrically, possess mechanisms of resistance against cytostatic drugs therapy and show a high capacity to repair DNA damage [3]. The origin of the CSC may take place in transformed dedifferentiated adult cells [128] or in cells that acquire progenitor or differentiated stem cell biological characteristics [15].

The previously accepted tumoral model (stochastic) states that most tumor cells are unstable genome carriers, prone to mutations that confer a competitive advantage and capacity of proliferation to the tumor cell, yielding a collection of varied cellular phenotypes. Tumor cells in this model are responsible for the survival, spread, resistance and metastasis [28, 54]. In short, the CSC is a cell capable of giving rise to neoplastic disease, and to be self-perpetuating, through different asymmetric division and differentiation, originating several cell lines that form the tumor mass [157]. This capability is demonstrated experimentally by the ability of these cells to give rise to new tumors in immunodeficient hosts in successive retransplantation, restoring tumor heterogeneity [126].

13.2 CSC and Gynecologic Cancer

Malignancies of the female genital tract constitute approximately 20 % of visceral cancers among women. This is the sixth leading cause of death in women. Siegel and collaborators (2012), estimated an incidence around 9 % of these malignancies in developed countries in 2012 (6 % body of the uterus and 3 % of the ovary) [132]. Those authors also estimated for 2012 mortality rates of 6 % for ovarian cancer and 3 % for uterine cancer. Approximately 80 % of gynecologic cancers are detected at advanced stages, usually with peritoneal and visceral metastasis [85] which determines a high mortality rate.

13.2.1 Ovarian Cancer

Ovarian cancer is the second most common gynecologic malignancy and the most common cause of gynecologic cancer death in the United States [132]. The majority of ovarian malignancies are derived from epithelial cells; the remainder arise from other ovarian cell types (germ cell tumors, sex cord-stromal tumors) [83].

In Europe, ovarian cancer was responsible for near 42,000 deaths in 2008 [39]. The 5 years survival rate is less than 45 % [164]. From the survivors, 80–90 % are diagnosed in stage I and less than 5 % in stage IV [64].

The main risk factors for epithelial ovarian cancer are those that increase the number of ovulations during a woman's life, such as nulliparity and hormone replacement therapy. Other epithelial ovarian cancer risk factors are endometriosis, polycystic ovary, familiar history of ovarian cancer, BRCA 1 and BRCA 2 mutations. Protective factors are the ones that decrease the number of ovulations in women as delayed menarche or early menopause [122], multiparity [145], lactation, and the use of oral contraceptives [106].

The role of the ovarian epithelium in carcinogenesis was first recognized in 1872 by Spencer Wells [62]. Although the ovarian surface epithelium represents a small part of the cell mass of the ovary, it represents the source of 90 % of all ovarian cancers [106]. Murdoch and Martinchick [105] supported that the continuous and repeated rupture and wound healing of the ovarian surface, during ovulation, was the initiator of neoplastic transformation. The biochemical and inflammatory events, the presence of tumor necrosis factor, the epithelium injury and wound healing in an environment of continuous hormonal cyclical modifications, are continuously happening in the ovary of the fertile female. The lesions caused during ovulation start to heal and the defect is filled by regenerative cellular replication, through stem cells and cell migration from the wound margins. The integrity of DNA in cells near the site of rupture is compromised during all this process. Thus, repeated episodes of wound-healing-hormonal changes, referred previously, could lead to expansion of a cell clone, with altered genetic, epigenetic and morpho-functional integrity, resulting in a carcinogenesis process [106]. It is precisely the presence of stem cells in epithelial surface that allows the regeneration of ovarian surface epithelium

and the proper healing of the lesion through asymmetric cell proliferation [137]. Auersperg et al. [8] showed that the epithelial surface of the ovary and the fimbriae of the fallopian tubes constitute part of the same embryonic transitional epithelium. The tumors originated in this cells share common events in carcinogenesis, as well as clinical manifestations and prognostic value. The epithelial ovarian carcinoma metastasizes directly to adjacent organs or through peritoneal fluid in cellular spheroids [85]. These spheroids are elements of chemoresistance and spread of ovarian cancer because they transport CSCs and other cells associated with cancer development, such as fibroblasts, endothelial cells and inflammatory cells. The papillary serous carcinoma of the ovary will rise in endometrial intraepithelial carcinoma foci, in multifocal noninvasive neoplastic disease of the uterus, in less cohesive cells with ability to migrate and expand through the peritoneum that will set definitely on the surface of the ovary or fallopian tubes [96]. Prognosis of ovarian carcinoma is usually better in young women [18] with good performance status and lower tumor burden at diagnosis. Risk factors for poor prognosis are: the histological clear cell subtype [148], the persistence of high levels of Ca125 after surgery [107], high histological grade, the presence of ascitis and the rupture of the capsule [51].

13.2.2 Uterine Cancer

Uterine cancer is the most common gynecologic malignancy in developed countries, with an incidence of 12.9 per 100,000 women and a mortality rate of 2.4 per 100,000. In developing countries, it is the second most common gynecologic malignancy (cervical cancer is more common), with an incidence of 5.9 per 100,000 and a mortality rate of 1.7 per 100,000 [70] Worldwide in 2008, near 290,000 women were diagnosed with uterine cancer [70]. Adenocarcinoma of the endometrium (lining of the uterus) is the most common histologic site and type of uterine cancer.

The type 1 of endometrial cancer (endometrial adenocarcinoma) is the most common type and affects mostly pre and post-menopausal women. It is often related with endometrial hyperplasia. Usually it has a favorable prognosis and good clinical response to treatment with estrogens.

The type II of endometrial carcinomas (serous, and clear cells) occurs in post-menopausal women, has no clinical relationship with estrogen and usually has a worse prognosis.

Risk factors for endometrial cancer are those which provide an overexposure to estrogens, non-compensated by progesterone (hormone replacement therapy during menopause without progestins), tamoxifen therapy, obesity and nulliparity.

The cyclical process of regeneration, growth and endometrial loss happens more than 400 times in a woman's life [82]. This cyclical event implies a high regenerative capacity. The presence of SC in this highly proliferative tissue was confirmed, as well as in endometrial adenocarcinoma [44, 75] demonstrated the existence of CSCs in endometrial carcino-sarcoma, in vivo and in vitro.

Kato [72] defines the fundamental characteristics of the CSC in endometrial carcinoma: reduced expression levels of differentiation markers, long-term repopulation properties, self-renewal capacity, and enhanced migration, enhanced tumorigenicity and asymmetrical division.

13.2.3 Cervical Cancer

In 2008, cervical cancer accounted for 9 % of the total new cancer cases and 8 % of the total cancer deaths among females worldwide [70]. The incidence and mortality is higher in developing countries due to lack of implementation of screening programs and lack of HPV vaccination [165].

Human papillomavirus (HPV) is the cause of cervical cancer and can be detected in 99.7 % of cervical cancers [152]. The most common histologic types of cervical cancer are squamous cell (69 % of cervical cancers) and adenocarcinoma (25 %) [121].

Probably the origin of cervical carcinoma is in Side Populations (SP) with biological characteristics of CSCs [150], located in subcolumnar reserve cells [65].

The most relevant markers of CSCs in cervical carcinoma are Nanog, Nucleostemin and Musashi1 [158].

In spite of the clear gynecological cancer advances in surgical and cytostatic therapy, high rates of relapse and chemoresistance persist. This high rate of relapses indicate that current strategies are insufficient to cure the disease. A probable cause of this failure is the inability of these therapies to destroy CSCs (these cells have biological characteristics that makes them immune to chemotherapy). The definition, identification of the CSC and its membrane markers will define therapeutic targets that will allow the elimination of these cells and improve the prognosis of gynecologic cancer [111].

13.3 Identifying Cancer Cells with Stem-Like Characteristics in Gynecologic Cancers

13.3.1 CSC Markers: Historical Overview and General Concepts

Tumors are formed by heterogeneous populations of cells and understanding the origins and roles of these different cell types is essential to develop prevention strategies, and future tools for diagnosis and therapeutics. Small side population (SP) cells, enriched in stem-like cells, have been identified in several tissues and tumors based on their ability to efflux the fluorescent dye Hoechst 33342 [72–74]. When present in tumors, these stem like SP are currently referred to as Cancer Stem Cells (CSC) [141].

According to the American Association of Cancer Research, CSC is defined as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor" [27]. This definition did not consider the source of the cells, which led to the necessity of creating complementary terms such as "tumor-initiating cell" or "cancer-initiating cell". It has been proposed that these CSC may arise from normal cells including untransformed stem cells, progenitor cells, mature cells, and cancer cells that somehow suffer genetic/epigenetic alterations [115].

Up to recently, the idea of CSC still raised some controversy [60] and similarly, the CSC model regarding cancer development, despite being currently widely accepted, has faced severe criticism. Also, as highlighted by Fabian et al. [37], in spite of the enormous efforts to isolate pure CSC populations, it has not yet been fully achieved. Moreover, frequently cell populations with some contradictory surface marker signatures show clonogenic and tumorigenic potential. This is certainly due to the still limited technical advances in CSC isolation and characterization. Currently, the isolation of CSC is often done by (a) isolation by sorting of a SP, based on Hoechst dye efflux; (b) sorting on the basis of cell surface marker, or (c) sphere culture [114].

CSCs were described for several human cancers including breast [112], colon [120], head and neck [117], gastric [142], lung [35], melanoma [38], pancreatic [66], prostate [112] and others. Considering the gynecologic cancers, CSC were also reported for ovarian (e.g., [14, 163]), endometrial (e.g., [23, 44, 49]) and cervical cancers (e.g., [156]).

According to the growing evidence of CSC presence in a large number of cancers, and their increasing correlation with cancer development, and with treatment resistance and recurrence mechanisms, the model of CSC is gaining acceptance among scientific community. This model suggests that the malignancies associated with cancer originate from a small population of stem-like, tumor-initiating cells.

In the CSC concept model, a tumor shows a hierarchy analogous to the one observed in a normal tissue [151]. According to this model, and like normal stem cell populations, CSC are able to divide asymmetrically, yielding both an undifferentiated stem cell (that will self-renew) and a daughter cell that will differentiate. Differentiated tumor cells form the bulk of the tumor mass, but are unable to self-renew [82]. This repetitive process allows that, with time, cellular alterations will accumulate [115].

CSC are classically supported by: (i) their capacity to self-renew; (ii) their ability to give rise to a heterogeneous progeny of cells; and (iii) their ability to modulate their differentiation and self-renewal according to genetic and environmental controls [5, 115].

The hypothesis that CSC may initiate and sustain tumor growth supports a cell hierarchical organization in the tumor and is supported by the fact that CSCs induced tumors formation in serial xenotransplantation assays, being able to reestablish the hierarchical cell organization and heterogeneity of the parental tumor. Therefore, it is hypothesized that CSCs have key roles in cancer development and progression [5, 141].

The development of molecular cell markers for specific CSC is of crucial importance for separating these cells, and developing targeted therapies against CSC. However the development of reliable molecular CSC markers remains highly complex under debate [11, 37]. Membrane markers of normal stem cells in the organ provide an interesting guideline for examining the CSCs present in tumors of that organ [11].

Some of the most commonly used cell surface markers to detect CSC include CD133, CD44 or CD24, CD90, CD34, CD117 or CD20, while functional assays include aldehyde dehydrogenase (ALDH) or exclusion of Hoechst dye [37, 98]. It should be noted that the role and reliability of some of these markers remains controversial, including the CD133 [37].

Disruptions in several cellular signaling pathways, including Wnt, Hedgehog, Notch, Nanog or transforming growth factor beta (TGF-β) cellular pathways, as well as in ABC multidrug transporter signaling pathways (within the ATP binding cassette transporters family), have been identified in cancers and in CSC (e.g., [4, 36, 101]).

Some members of these pathways/cascades are being proposed as putative markers of these CSC or as potential targets for therapeutics. However, it still remains to unveil the genetic and epigenetic regulations of these complex pathways in CSC [29, 102]. Additionally, and despite a plethora of potential markers, it is important to retain that the molecular markers currently available are not exclusive of the CSCs, and that often a cell population negative for CSC markers may also exhibit potential for tumor initiation [37]. For example, it was found that, occasionally, cells expressing either CD133+ or CD133- may have CSC characteristics, and that expression of CD133 is affected by gene methylation.

Currently it is being studied how pathways involved in differentiation of normal stem cells that are deregulated during carcinogenesis are involved in stemness-associated pathways.

13.3.2 Markers of CSC in Gynecological Cancers

13.3.2.1 Ovarian Cancer Stem Cells (OCSC)

Overall, ovarian cancers are associated with a lack of early symptoms, although being extremely aggressive and having rapid progression leading often to poor prognosis for patients [14]. As other cancers, ovarian cancers also present SP with characteristics of ovarian cancer stem cells (OCSC) [115].

Different profiles of abundance were described for OCSC in different cell lines. For example, OCSC are rare in some human ovarian cancer cell lines including SKOV-3, IGROV-1, OVCAR-3, being more frequent in the murine ovarian carcinoma *line* MOVCAR7 [141]. OCSC have stem-like characteristics, and have abilities of colonizing, unlimited self-renewal/proliferation and to differentiate into heterogeneous tumors. In the last years several studies aimed at characterizing

OCSC [20]. Bapat et al. [14] isolated and characterized 19 spontaneously immortalized clones that expressed cell surface markers such as c-kit, stem cell factor, CD44, EGFR, and E-cadherin. Two of these clones also exhibited anchorage independent growth and were able to induce xenografted tumors in nude mice and expressed the stem cell factor CD117, representing the first isolation and characterization of stem-like cells in ovarian cancer.

The octamer-binding transcription factor 4 (Oct4) is an important embryonic transcriptional factor, being highly expressed in several tumors and is considered as a hallmark of cancer stem cells [88]. This gene Oct4 is often reported as a "stemness gene".

Gao et al. [47], using qRT-PCR, revealed that in the human ovarian cancer cell line OVCAR-3, the SP cancer cells expressed higher levels of Oct4 than the other cancer cells, supporting that these SP cells were rich in a CSC population. Therefore, increasing knowledge about the expression and regulatory mechanisms of Oct4 will provide crucial information on how OCSC regulate ovarian cancers, and may be an important target of future therapeutic approaches.

Besides Oct4, cellular markers for OCSC may imply the use of undifferentiated stem cell markers such as Nanog (a transcription factor), BMI1, or ABC transporters (e.g., ABCG2), combined with ovarian specific markers and functional assays [11]. This author reviewed the importance of cell membrane markers in OCSC such the CD133+; CD44+/CD117+ (first reported by Zhang et al. [162, 163]), CD44/MYD88+ (also descried by Alvero et al. [5]), besides the functional exclusion of Hoechst 33342 (through ATP-binding cassette transporters).

However, the use of OCSC markers is not yet absolutely reliable, probably due to the fact that ovarian cancer is a complex malignancy, grouping different types of diseases. For example, Theriault and Shepherd [143] stressed that despite some e*pithelial ovarian carcinoma* (EOC) biomarkers including CA125, mesothelin and HE4 are available, the identity of reliable markers that differentiate OCSC from the rest of the tumor population remains uncertain. In particular the authors implicated CD133 as a potential, but not obligate, marker for OCSC. Ferrandina et al. [41] also demonstrated that the immunohistochemical assessment of CD133 did not provide information of clinical value for prediction of response to treatment or prognosis, in ovarian cancer patients.

Recently, Burgos-Ojeda et al. [20] proposed that ALDH and CD133 could be used to identify distinct chemoresistant ovarian CSC populations, and both $ALDH^{+}CD133^{+}$ cells and $ALDH^{+}CD133^{-}$human ovarian tumor cells could initiate tumors in mice. Additionally, and as emphasized by Gallagher et al. [46], different genetic profiles are employed by primary and recurrent ovarian tumors. Zhang et al. [163] used primary tumor specimens to obtain non-adherent cells (which formed spheroids resistant to conventional chemotherapy) that were identified by CD44+/CD117+ markers.

Reduced ALDH1 expression was found to be associated with malignant transformation in ovarian cancer [133]. Moreover, SP cells (enriched in CSC) express high levels of various members of the large family of ABC-transporters, including MDR1 and BCRP that belong to the family of multidrug resistance proteins

(MDR). So, interesting strategies to improve cancer therapy may include blocking these transporters [61]. Additionally, the Hedgehog (Hh) pathway seems to play a crucial role in regulating growth of ovarian cancer spheroid-forming cells [118], despite further studies are needed to clarify the changes that occur this pathway in OCSC.

Mor et al. [103] reviewed the recurrence and chemoresistance of EOC, based on the classification of Type I and Type II EOCSC. Type I was selected by the cell surface marker CD44, was chemoresistant, had constitutive NFκB activity and constitutively secreted cytokines as IL-6 and chemokines (e.g., *IL-8*, *MCP-1)*. Type I and Type II EOC cells showed several differentially expressed genes. In fact, CD44, MyD88, Oct4, Snail 1, Sox 2, Klf4, IGFBP7, RhoE, Rac2, PML1, PML2, CK19, ALDH1, EpCAM, β-catenin, Nanog, CK-18 and L1-CAM were reported as stem cell-associated genes in Type I EOCSC [103]. The authors supported that Type I EOC may represent the subpopulation that has stem-like properties. The overexpression of mucin-4 (MUC4) mRNA has also been reported in ovarian cancer and [116] demonstrated that MUC4 overexpression also led to an enriched OCSC population proposing a MUC4-directed therapy for OCSC.

Anderson et al. [7] reported that alterations in Bcl-2 expression (an important protein controlling mitochondrial external membrane permeability and apoptotic cascade) could be correlated with ovarian cancer progression. Also, two members of the HOX gene family (it regulates cell-fate specification to govern identity of body segments), namely HOXB4 and HOXB7, showed higher expression in ovarian cancer cell lines compared to the normal, while HOXB13 enhanced the proliferation of the human ovarian cancer cell lines SKOV-3 and OVCAR5 and of a mouse ovarian cancer cell line [76]. These data confirm the involvement of HOX members in the development of ovarian cancer.

In a genomic approach of ovarian cancer cells, Vathipadiekal et al. [146] used microarray analysis to demonstrate an expression profile for SP enriched for OCSC. The "stemness" gene signature nature of this SP was established by the identification of several stem cell-related genes (e.g., activated Notch signaling pathway). Gallagher et al. [46] stressed that genes involved in a "stemness signature" have high importance in processes related with cell proliferation and apoptosis and presented a p53-p21 cancer stemness signature model for ovarian cancer. Apparently, CSCs survive to chemotherapy and contribute to recurrence by using different mechanisms than those used by the primary disease.

Understanding the signaling signatures related to cell plasticity in the multiple steps of ovarian cancer progression is an important issue to better understand and fully control the multiple pathways involved in this malignancy.

13.3.2.2 Endometrial Cancer Stem Cells (ECSC)

According to the CSC concept model, genetic alterations arising in endometrial stem cells (ESC) located in the basal layer remain and are passed on to next generations of cells that may even accumulate additional genetic alterations [82] and may

be at the basis of endometrial carcinogenesis. Few studies have focused on providing evidence about the existence of endometrial cancer stem cells (ECSCs) and their role in endometrial cancer development (e.g., [23, 44, 48, 49]. For example, Friel et al. [44] showed that a SP fraction from the endometrial cancer cell line An3CA had characteristics of CSC. Hubbard et al. [68] demonstrated that SP cells isolated from primary endometrial tumors showed self-renewing, differentiating and tumorigenic properties, so the authors hypothesized that CSC are involved in endometrial carcinoma development.

The analysis of genetic alterations in the some cellular pathways involved in endometrial tumors (e.g., Notch, PI3K/AKT, Wnt/β-catenin) has shown to be highly informative for clinical use. Similarly, Gotte et al. [53] found that the RNA binding protein Musashi-1 (a RNA-binding protein associated with maintenance and asymmetric cell division of epithelial progenitor cells) was expressed in a subpopulation of endometrial cancer cells. Specific cell pathways (e.g., Notch) may be interesting targets to limit proliferation of endometrial CSC.

Therefore, modulating endometrial carcinoma cell cycle progression and apoptosis via the stemness-related factors using those specific targets may have evident potential in future endometrial carcinoma therapy.

Hedgehog and Wnt signaling pathways are recurrently pointed as playing important roles in human gynecological cancers. For example, aberrant activation of the Hedgehog (Hh) signaling pathway, and its susceptibility to transcriptional factors regulation, was found in endometrial carcinogenesis [86]. These results support that new drugs targeting genes related with these pathways may hold clinical potentials of being diagnostic biomarkers.

An association between the oncogene BMI1 (B lymphoma Mo-MLV insertion region 1 homolog) and enhanced stem cell proliferation was established, supporting that BMI1 expression may be required for regulation of stemness properties in EC cells [34]. Cervello et al. [22] described the most likely markers for endometrial somatic stem cells (Oct-4, Musashi-1, CD31, CD34 and CD144).

In two recent experiments, Wang et al. [153] reported that endometrial regenerative cells had high levels of IL-8 and ICAM-1 and also expressed CD9, CD29, CD41a, CD44, CD59, CD73, CD90 and CD105, while [102] confirmed that CD133+ cells may contribute to endometrial cancer tumorigenicity and recurrence.

The pathways underlying the CSC involvement in endometrial carcinoma development remain unexplored. It should be highlighted that hyperactivation of Hh and Notch signaling pathways – both important in cell programming and determination – is frequently observed in ovarian, endometrial and cervical cancers. Also the overexpression of Nanog exerts a prominent effect in gynecological tumorigenesis. Understanding the involvement of alterations in these pathways in gynecological CSC may be a powerful tool in developing new pharmaceutical targets for gynecological malignancies. An increase of IPO13 expression was also reported in endometrial cancer [161], as well as other pathways that may also be involved. An example is the involvement of the histone deacetylase, as it was found that sodium butyrate (inhibitor of this enzyme) inhibited the self-renewal capacity of endometrial cancer SP cells [74].

LKB1 is a significant tumor suppressor in the lung and was the first gene that was found to be mutated in a significant proportion of cervical cancers. Interestingly, the majority of cervical cancer cell lines, including HeLa, harbor homozygous *LKB1* deletions [156]. Type I endometrial cancers were often correlated with disorders in pathways involving mutations in the genes including the *PTEN, MLH1, MSH2, MSH6* (DNA mismatch repair genes), *KRAS* and/or *CTNNB1* (β-catenin) [153]. The Wnt/β-catenin and PTEN signal transduction pathways (involved in adult stem cell self-renewal and maintenance) suggest a stem cell origin of the cancer [75]. In particular, Slomovitz and Coleman [134] stressed that, among others, a loss of PTEN may be indicator of sensitivity to PI3K/AKT/mTOR pathway inhibition, while activating KRAS mutations may predict resistance. This is particularly important as an overactivation of the PI3K/AKT/mTOR pathway (a signaling pathway in cellular growth and survival) has recently been implicated in endometrial cancer pathogenesis, supporting that research to inhibit this pathway is of therapeutic interest [134]. In Type II endometrial cancers, the SP enriched with CSC showed often mutations in *TP53* and *ERBB2* [154].

Additionally, in endometrial carcinoma, the percentage of cells expressing the RNA-binding protein Musashi-1 is high if compared to healthy cells. Despite its role in endometrial cancer induction and cancer progression is currently unknown, it is being reported as a stem cell marker for endometrial carcinoma [52, 53, 91]. Its role in CSC may be related with abnormal pathways of cell cycle progression and apoptosis, as Gotte et al. [52] demonstrated that Musashi-1 modulated pathways of cell cycle progression and apoptosis via the stemness-related factors Notch-1, Hes-1 and p21WAF1/CIP1 in endometrial carcinoma. These new findings place Musashi-1 as an interesting new target for regulation and open new perspectives for EC therapy.

Functional genome analysis is bringing important information to the still puzzling role of CSC in tumor development. Compared to normal tissue, endometrial polyps showed a decrease in the mRNA expression levels of IPO13, c-kit, telomerase, caspase3 and bax, while the expression of bcl-2 increased. The authors proposed that the development of endometrial polyps is associated with the deregulated activities of the endometrial stem/progenitor cells [67].

13.3.2.3 Cervical Cancer Stem Cells (CCSC)

Cervical cancer is the third most common carcinoma in women worldwide [159] but few studies address the characteristics of cervical cancer stem cells (CCSC) in cervical malignancies. Self-renewing subpopulations found among four well known human cancer-derived cell lines (HeLa, SiHa, CaSki and C-4 I) were shown to express characteristic markers of stem cells [89].

The regulatory pathways of CCSC remain to unveil, despite great efforts in research made by several groups in the last few years. The octamer-binding transcription factor 4 (Oct4) is highly expressed in several tumors but its role in the development of cervical cancer is still obscure. Liu et al. [88] demonstrated that

the human papillomavirus positive cervical cancer cells CASki showed higher levels of Oct4 than the virus free C-33A control cells. Contrarily the levels of histone deacetylase 1 and DNA methyltransferase 3A were lower in CaSki cells. In the same study it was demonstrated that treatment with valproic acid, an histone deacetylase inhibitor increased the expression of Oct4 in C-33A cells, but only slightly increased Oct4 in CaSki cells [88].

Cai et al. [21] identified a cell cycle regulatory pathway in which the miR-302-367 cluster down-regulated both cyclin D1 and AKT1 and up-regulated p27 and p21, suppressing cervical cancer cell proliferation. As consequence the authors proposed that the miR-302-367 cluster could be used as a therapeutic reagent for the treatment of cervical cancer.

Hyperactivation of Hh and Notch pathways have been frequently observed in gynecological malignancies including cervical cancers. In contrast, the expression profiles of pluripotency-regulating transcriptional pathways – Nanog, Oct4 and Sox2 – appear heterogeneous [93]. Also mesenchymal stem cells showed the expression of Nanog in the cervical cancer, which supports the discussion around the use of this transcription factor as a cervical cancer progression marker [57].

The expression of other proteins and putative correlation with cancer development and stem cancer cells is being largely studied. For example, the protein Nestin was correlated with aggressive growth, metastasis, poor prognosis and presence of CSC in various tumors. Nestin plays important roles in carcinogenesis and tumor formation of cervical cancer cells [127] but its role in CCSC requires further elucidation. Su et al. [140] found that the protein PTPRR (protein tyrosine phosphatase receptor type R) was silenced through a DNA methyltransferase mediated methylation in cervical cancer. The authors concluded that methylation of the PTPRR promoter has an important role in the metastasis and may be a biomarker of invasive cervical cancer.

The advances from the last years demonstrate that, as other cancers, cervical cancer development involves cellular alterations including changes in multiple proteins and pathways. Understanding the upstream regulatory mechanisms is an important issue to understand the development of cervical cancer and to contribute to the development of new therapies.

13.3.3 Genetic/Epigenetic Regulation in Gynecological Cancers

Epigenetic plasticity refers to the capability of cells to alter their phenotype as a result of changes in mechanisms other than those related with DNA sequences, namely alterations associated with chromatin remodeling [13] These changes occur as responses to cell microenvironment changes, but their roles in cancer development remain unclear.

Recently genome/epigenome analyses were performed that may contribute to further deciphering the genomes and epigenomes of gynecological CSC [9, 50, 160]. For example, p53 mutations/inactivation seems to have various profiles among the

different types of OC. While p53 mutations/inactivation play a role in epithelial OC, granulosa cell OC were found to have intact p53 but often unregulated PTEN and Wnt/β-catenin signaling [43]. It remains unclear how epigenetic events contribute to these different pathways regulation in OC different types. On other hand, some understanding was provided for epigenetic regulation in EC by Dellinger et al. [31] who showed that the (de)regulation of the Wnt/β-catenin signaling pathway in EC was controlled by epigenetic events.

The involvement of epigenetic alterations may occur early in carcinogenesis and even may mediate transformation. It is known that CpG hypermethylation plays an important role in cancer development and therefore their use as epigenetic markers has been proposed [19]. Data on CpG island hypermethylation provided complementary information on the different ovarian cancer types and on cancer evolution [129]. For example, loss of DNA methylation as well as gain of promoter-associated CpG methylation, were associated with all stages of EOC.

Epigenetic alterations can also be due to histone modifications as demonstrated for different OC lines [11]. Berry and Bapat [19] stressed that CSCs's differences from the parental tumors may be explained by early epigenetic changes. Both mutagenesis and epigenetic events may therefore further facilitate CSC aberrant functioning. Recently Bapat [12] integrated genomic and epigenomic (including miRNA) assessments to reveal a complex network of epigenetic mediated regulation, influenced by the cellular microenvironment, which support the epigenetic plasticity of ovarian cancer.

Many of the above referred chromatin modifications are repressive, and act to silence tumor suppressor genes often associated with modifications in bivalent domains in/of histone. Several histone modifications regulate gene expression by involving factors that modify chromatin fiber compaction. Histone methylations at lysine and arginine residues are another class of epigenetic marks. Trimethylation of H3K4 and monomethylations of H3K9, H3K27, H3K79, H4K20 and H2BK5 are linked to gene activation, while trimethylation of H3K27, H3K9 and H3K79 are related with repression. Also, bivalent epigenetic marks (H3K4me/H3K9me2 and H3K4me/ H3K9me3) were associated with CSCs [108].

DNA methylation events have been proposed as prognostic markers. Fiegl et al. [42] analyzed the DNA methylation of 71 genes in 22 ovarian cancers and 18 non-neoplastic samples and identified the genes of the human homeobox genes family (HOX), respectively HOXA10 and HOXA11, as the best discriminators between cancer and non-neoplastic tissues. In particular HOXA11 methylation was strongly associated with poor outcome, suggesting a possible role for DNA methylation as a prognostic marker in ovarian cancer. Also, increased expression of HOXA10 is present in ovarian carcinomas as a result of promoter hypomethylation of HOXA10 [26]. This could also act as a prognostic marker in ovarian cancer as well as a possible therapeutic target, for example by using drugs that can reverse epigenetic changes.

Friel et al. [45] suggested that CD133 expression was epigenetically regulated in cells from primary human endometrial tumors. This was later supported by Min et al. [102]. These authors used ovarian cancer cell lines (OVCAR-8 and IGROV-1)

and an endometrial cancer cell line (Ishikawa) treated with 5-aza-2'-deoxycytidine (DAC) or Trichostatin A (TSA) to demonstrate that the expression of CD133 in primary ovarian and endometrial cancer cell lines is regulated by epigenetic events. Also, the short non-coding microRNAs (miRNAs) hybridize at specific of the mRNA preventing translation and therefore exerting a post-transcriptional control. MicroRNAs (miRNAs) are a class of small non-coding RNAs and with a regulatory function in the cell [10].

Despite their unequivocal role in epigenetic control of cell division, differentiation and other metabolic processes the knowledge of the underlying networks controlled by miRNAs remain unclear. Iorio et al. [69] reported that several miRNA had differential expression in epithelial ovarian cancer tissue when compared with normal tissues. According to these authors the most significantly over-expressed miRNAs in ovarian cancer tissue were miR-200a, miR-141, miR-200c and miR-200b. On other hand, miR-199a, miR-140, miR-145 and miR-125b1 were the most down-regulated.

Specific miRNA populations have been described in stem cells, CSCs and cancers in general. For example, the oncogenic role of the miRNA family, miR-17/92, is well defined [33, 147]. It has also been reported that miRNAs located in specific clusters on chromosomes are often simultaneously synthesized, and the expression of miRNAs at chromosomes 14 and 19 has been linked to ovarian malignancy [100, 162].

13.4 Therapeutics Approaches in Gynecologic Cancer

13.4.1 Systemic Therapies

The therapeutic approach to advanced gynecologic cancer involves surgery, radiotherapy and chemotherapy.

Despite advances in these therapeutic strategies most patients with gynecological cancers relapse and become drug-resistant [46].

The conventional chemotherapeutic drugs usually target fast-dividing cells, but CSCs can survive those conventional chemotherapeutic treatments probably due to several biologics mechanisms. Some studies conducted on non-small cells lung cancer relapse show increase in CSCs, which strongly suggest that CSCs may contribute to disease relapse [139].

Therefore, a pathophysiological concept to develop alternative therapies specifically targeted to CSC has emerged as having high clinical interest [87]. The importance of gynecological CSC-targeting alternative approaches was summarized by Gallagher et al. [46] as "the most alarming aspect of CSCs is their uninhibited proliferation in the presence of chemotherapeutic agents".

Despite these biological properties of CSCs and their confirmed lower sensitivity to conventional therapies, define them as key objective in emerging medical therapies of gynecologic cancers, no specific target for CSC has yet been identified [2].

Concerning the ovarian cancer, Peracchio et al. [113] discussed the main factors that govern the recurrence and progression of ovarian cancer, which include the propensity to trigger a program of epithelial-mesenchymal transition, the overexpression of drug efflux transporters and the persistence of dormant cancer stem cells. Initially advanced ovarian cancers usually are responsive to standard chemotherapies (cisplatin and paclitaxel), but later they transform into a drug resistant phenotype [115]. These cancer cells showed to be more resistant to cisplatin and paclitaxel, suggesting a possible role for these cells in ovarian cancer chemoresistance [115]. Therefore, it is currently accepted that OCSC, through selective carriers may be key players of chemotherapy resistance acquisition, and of recurrence of ovarian cancer [102].

13.4.2 CSC and Emerging Therapeutic Approaches

13.4.2.1 Membrane Markers

CSCs are defined by several membrane markers which could be used as a therapeutic target and have demonstrated clinical response to treatment and improved prognosis in some patients.

CD44 is a cell surface transmembrane glycoprotein, involved in cell-cell and cell-matrix interactions, which affects cell growth, cell differentiation and motility [94]. It is present in primary and metastatic ovarian cancer [5] and its expression is associated with chemoresistance and a poor prognosis [99].

Several monoclonal antibodies have been developed targeting CD44v6 isoform (correlated with aggressive behavior of different cancer), with negative results in phase I clinical trials [1]. However, in another pharmacologic approach, a bioconjugate using hyaluronic acid (CD44 mediates cell adhesion and migration by binding extracellular matrix components as hyaluronic acid) [1] linked to paclitaxel is being developed to increase the influx of cytotoxic drugs in ovarian cancer cells that overexpress CD44, in the treatment of intraperitoneal ovarian cancer [30, 110] and data strongly support the development of this strategy for locoregional treatment of ovarian cancer.

CD133 is a transmembrane glycoprotein overexpressed in ovarian carcinoma, associated with poor prognosis [40]. The development of an antibody against CD133 presents the opportunity to eliminate a potentially drug-resistant cancer subpopulation [1] As well as for CD 44, for this membrane marker, a bioconjugate (resulting from the combination of monoclonal antibodies against CD133 and monomethyl auristatin F – MMAF – a potential cytostatic), has demonstrated *in vitro* efficacy in stomach and hepatocellular neoplasias inducing apoptosis [135]. The authors conclude that Anti-CD133 antibody-drug conjugates warrant further evaluation as a therapeutic strategy to eradicate CD133+ tumours.

CD117 (c-kit) is overexpressed in 40 % of ovarian tumors and is correlated with resistance to classical cytostatic therapy [92]. The expression of CD117 confers

CSC-like characteristics to cells [92, 163]. Imatinib mesylate combined with Paclitaxel and Carboplatin has shown response in cellular studies [104]. It was demonstrated that some responses were maintained or the disease remained stable when, in clinical trials, it was combined with docetaxel for patients previously treated that presented platinum-resistant tumors or recurrent disease [97].

CD24 is a cell surface protein expressed in hematologic diseases and in some solid tumors. There is a statistical association between CD24 overexpression and invasive ovarian carcinomas [16, 79].

Gao et al. [47] have demonstrated that CD24 is a phenotypic marker of ovarian CSCs. CD24+ cells are resistant to chemotherapy, but are sensitive to lysis by NK cells. Therefore, immunotherapy with NK cells may be a future strategy for the destruction of ovarian CSCs and prevention of tumor relapse and metastasis [78].

EpCAM: Epithelial Cell Adhesion Molecule (EpCAM; CD326), is a glycosylated membrane protein, with signaling properties which mediate the tumor's formation and proliferation [28]. It is overexpressed in ovarian tumors and is marker for poor prognosis [138]. It is clearly overexpressed in recurrences and metastases of ovarian cancer [17]. Several anti-EpCAM antibodies have been developed.

Catumaxomab (trifunctional monoclonal antibody anti-EpCAM x anti CD3) has been approved by EMA for the treatment of ovarian cancer in patients with malignant ascites, with favorable results [130].

Adrecolomab [119] and adecatumumab have not demonstrated positive clinical outcomes [81].

Adecatumumab is a fully human recombinant antibody that targets EpCAM positive ovarian cancer cells and has shown potent antitumor activity in ovarian cancer in vitro and in vivo [32].

13.4.2.2 Cell Differentiation

Another therapeutic approach for gynecologic CSCs is the pharmacological induction of cell differentiation and the elimination of the cellular ability of self-renewal [1].

Differentiation therapy pursues the discovery of novel molecules to transform cancer progression into less aggressive phenotypes by mechanisms involving enforced cell transdifferentiation.

Specific unsatturated fatty acids, such as palmitoleic, oleic and linoleic acids, trigger phenotypic modifications in a large number of human cancer cell lines (HCCLs), including ovarian carcinoma SK-OV-3 resulting in the transdifferentiation of HCCLs into adipocyte-like cells [123].

Retinoic acid and its derivatives are the only differentiating agents used to date, with proven benefits in acute promyelocytic leukemia, but have not shown clinical effectiveness in solid tumors [136].

HDACs I (Histone deacetylase Inhibitors): Sodium Butirate (NaB) [95] have demonstrated that regulation of histone acetylation is involved in the control of cell differentiation and CSCs phenotype. HDAC inhibitors also mediate multiple biological effects, such as growth arrest, apoptosis, senescence, reactive oxygen species facilitaded cell death, mitotic cell death and antiangiogenesis.

Kato [72] has demonstrated that sodium-butirate reduces the self-renewal capacity and tumorigenicity of endometrial carcinoma cell lines in vitro.

13.4.2.3 Anti-epigenetic Alterations

Several epigenetic changes present in CSCs may have unequivocal interest for diagnostic and prognostic approaches, as they may be reversible by epigenetic therapies [129]. Therefore, the use of drugs against aberrant epigenetic modifications can be a good complementary treatment to limit CSCs proliferation. Emerging clinical trials are being developed using epigenetic modifying drugs associated with classical cytostatics.

A major target may be the gene Oct4 that encodes a transcription factor critical for the maintenance of pluripotency and self-renewal in embryonic stem cells [71], and was sown to be involved in ovarian cancer progression and chemoresistance [125]. Also [71] documented a novel designer zinc finger protein (ZFP) that could upregulate the endogenous Oct4 promoter in ovarian cell lines carrying a silenced gene. The authors also proposed that this new ZFP could be used for the epigenetic reprograming of cancer cells. This is clearly an emerging and largely unexplored field that urges further studies targeting epigenetic regulation in gynecological CSC.

13.4.2.4 Metabolism Modifiers

ALDH1 (Aldehyde Dehydrogenase 1) has recently been identified as a marker for OCSCs [84], but the role of this prognostic marker for ovarian cancer remains controversial. While Chang et al. [24] supported that the overexpression of this marker correlated with good prognosis. Wang et al. [155] correlated it with poor prognosis.

The association of ALDH1 or CD133 with CD44 confers poor prognosis to the disease [80, 133].

Pathways involving ALDH may be therefore a target for new therapeutic approaches. Burgos-Ojeda et al. [20] found in vitro that disulfiram is preferentially toxic to $ALDH^+$ OCSC and that this drug is highly synergistic with cisplatin therapy against OCSCs.

13.4.2.5 Mitochondrial Induced Apoptosis

The discovery of a drug that specifically targets the mitochondria to induce apoptosis opens a new venue for treating ovarian cancer. Compounds such as resveratrol (3,5,4-trihydroxystilbene) induced death in five human ovarian carcinoma cell lines by stimulating apoptosis including mitochondrial release of cytochrome *c*, formation of the apoptosome complex and caspase activation, but also by stimulating autophagic death [109]. However, the authors did not characterize the effects on OCSC.

More recently, some studies have focused attention on identifying compounds that specifically target gynecological CSCs by affecting their mitochondrial

function. In fact, OCSCs have consistently been reported as exhibiting resistance to apoptotic cell death induced by chemotherapeutic drugs, which supports the urgency of developing other effective and specific therapeutic approaches. For example, N-t-boc-Daidzein inhibited in vitro OCSC growth and viability and induced apoptosis by activating caspases and inducing mitochondrial depolarization [55]. The authors supported that this compound deserves further attention as a promising chemotherapic targeting OCSCs. Also, Alvero et al. [6] demonstrated that targeting mitochondrial bioenergetics is a potent stimulus to induce caspase-independent cell death in OCSCs. The authors exposed cells to the isoflavone derivative, NV-128, that depressed mitochondrial function (leading to decreased ATP levels) and increased mitochondrial reactive oxygen species formation. This OCSCs mitochondrial impairment activated the AMPKα1 pathway and the mitochondrial MAP/ERK kinase/extracellular signal-regulated kinase pathway in these cells, opening perspectives for new therapeutic approaches of OC.

The studies on specific drugs targeting endometrial and cervical CSC remain unknown. Berberine had anti-proliferative effects on HeLa cells and decreased the Bcl-2/Bax ratio, together with a release of cytochrome c from mitochondrion. Berberine also up-regulated Fas, FasL, TNF-alpha and TRAF-1 suggesting that this induced apoptosis involved death receptor pathways [90]. Despite using the highly abnormal model system, these results opened a perspective for human cervical carcinoma therapy including targeting cervical CSCs.

13.4.2.6 Nanoparticles Therapy

Therapeutic Nanoparticles –TNPs – are therapeutic agents associated with a drug-delivery molecule that have advantageous drug location, delivery and concentration at the target. Some are already being employed in ovarian cancer treatment, such as **pegylated liposomal doxorubicin** (which uses a pegylated liposome as carrier) or polymeric carriers, such as **Xyotax (or CT-2103)**, poly-L-glutamic acid and paclitaxel [25], and **IT-101**, *cyclodextrin*-based *polymer* containing topotecan. These compounds present smaller clearance and a great half-life, being a more efficient drug delivery.

Xyotax has shown a better clinical efficacy than paclitaxel in previously treated patients, although with higher neurotoxicity [124]. Aguilar-Gallardo et al. [1] summarize the role of TNPs targetting CSCs, with molecules and complexes that surpass barriers for intratumoral and intracellular delivery of drugs, thus avoiding multidrug acquired resistance and enhancing the pharmacokinetic characteristics of the cytotoxic drug. Therefore, more effective therapeutic dosages with few adverse effects are attained.

13.4.2.7 Others

Another promising treatment alternative of ovarian cancer is the activation of nuclear “peroxisome proliferator-activated receptors” (PPARs). These receptors,

that act in lipid and glycidic metabolisms, regulate cell proliferation (PPAR-gamma), death, and cell differentiation in many tissues [56].

The association of PPAR-gamma ligands may be beneficial in chemoprevention and adjuvant therapies in some tumors [77].

PPAR-gamma was expressed in a large number of epithelial ovarian tumors and cell lines. The PPAR-gamma ligand ciglitazone inhibited the growth and clonogenic survival of ovarian cancer cells, inducing cell cycle arrest and cell death. Vignati et al. [149] says that PPAR-gamma activation by selective agonists is a valid strategy for ovarian cancer therapy and prevention, and should be tested alone and in combination with other anticancer drugs.

Interleukin-6 (IL-6) is a multifunctional α-helical cytokine that regulates cell growth and differentiation of various tissues, which is known particularly for its role in the immune response and acute phase reactions. Cells of relapsed ovarian tumors resistant to chemotherapy present an overexpression of IL6 [47], and in vitro treatment with the IL6 inhibitor **Siltuximab** has been shown to sensitize cells to paclitaxel than were originally resistant, but combination therapy with siltuximab did not have a significant effect on paclitaxel resistant tumor growth in vivo [59].

13.5 Conclusions

In spite of the enormous preclinical and clinical advances in gynecological cancers treatment, cancer resistance and recurrence has remained a puzzling problem from the perspective of cell biology. The CSC conceptual model may explain most of these cell patterns, and elucidate some aspects of the multiple heterogeneity founds in gynecological cancers. Understanding gynecological CSC morphology, function and roles in cancer development, resistance to therapy and recurrence is probably the major challenge in gynecological cancer research for the next decade.

Research on gynecological CSC is recent and is a crucial emerging field. For the last years, most focus was given on developing adequate techniques of CSC isolation, and the CSC characterization and selection of adequate molecular markers. Also, identifying both the most crucial signaling pathways (eg., Notch, PI3K/AKT, Wnt/β-catenin) altered in the different gynecological cancers and in their CSC subpopulations may provide additional evidence to understand CSC roles in tumor development and in therapy resistance. On other hand, targeting these altered pathways may provide novel tools for therapeutic approaches. This review highlighted most relevant markers and methodologies currently used to identify and isolate gynecological CSC advances in deciphering deregulated pathways. Finally, we also discuss some of the emerging strategies targeting gynecological CSC for potential therapeutic approaches, membrane markers, cell differentiation inducers, epigenetic changes modifiers, metabolism modifiers, mytochondria induced apoptosis promoters and nanoparticles therapies have been show as actual and future treatment strategies.

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