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Review

Interpreting epithelial cancer biology in the context of stem cells: Tumor properties and therapeutic implications

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Abstract

Over 90% of all human neoplasia is derived from epithelia. Significant progress has been made in the identification of stem cells of many epithelia. In general, epithelial stem cells lack differentiation markers, have superior in vivo and in vitro proliferative potential, form clusters in association with a specialized mesenchymal environment (the 'niche'), are located in well-protected and nourished sites, and are slow-cycling and thus can be experimentally identified as 'label-retaining cells'. Stem cells may divide symmetrically giving rise to two identical stem cell progeny. Any stem cells in the niche, which defines the size of the stem cell pool, may be randomly expelled from the niche due to population pressure (the stochastic model). Alternatively, a stem cell may divide asymmetrically yielding one stem cell and one non-stem cell that is destined to exit from the stem cell niche (asymmetric division model). Stem cells separated from their niche lose their stemness, although such a loss may be reversible, becoming 'transit-amplifying cells' that are rapidly proliferating but have a more limited proliferative potential, and can give rise to terminally differentiated cells. The identification of the stem cell subpopulation in a normal epithelium leads to a better understanding of many previously enigmatic properties of an epithelium including the preferential sites of carcinoma formation, as exemplified by the almost exclusive association of corneal epithelial carcinoma with the limbus, the corneal epithelial stem cell zone. Being long-term residents in an epithelium, stem cells are uniquely susceptible to the accumulation of multiple, oncogenic changes giving rise to tumors. The application of the stem cell concept can explain many important carcinoma features including the clonal origin and heterogeneity of tumors, the occasional formation of tumors from the transit amplifying cells or progenitor cells, the formation of precancerous 'patches' and 'fields', the mesenchymal influence on carcinoma formation and behavior, and the plasticity of tumor cells. While the concept of cancer stem cells is extremely useful and it is generally assumed that such cells are derived from normal stem cells, more work is needed to identify and characterize epithelial cancer stem cells, to address their precise relationship with normal stem cells, to study their markers and their proliferative and differentiation properties and to design new therapies that can overcome their unusual resistance to chemotherapy and other conventional tumor modalities.

Keywords: Cancer stem cell; Clonality; Niche; Progression; Carcinogenesis; Therapy

Contents

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1. Introduction

The vast majority of human malignancies arise from epithelial surfaces [1]. In a longitudinal study from Denmark, 56% of all human cancers were carcinomas of external epithelia (skin, large intestine, lung, stomach, cervix), 36% were carcinomas of internal epithelia (breast, prostate, ovary, bladder, pancreas) and 8% were sarcomas and leukemias [2]. Thus, in terms of incidence, carcinomas dominate the oncologic landscape. The epithelial surfaces from which they arise undergo constant remodeling and renewal, in a tightly regulated system that has been shown to involve a hierarchy of cells with differing proliferative capacities. Population renewal begins with the slow-cycling stem cell. This renewal is greatly expanded by the rapidly cycling transit-amplifying cell(s), and results in a highly organized collection of non-cycling, terminally differentiated cells (Fig. 1). This hierarchy has been shown to exist in most epithelia, including skin [3], cornea [4], gut [5–7], breast [8], lung [9], prostate [10] and liver [11]. It is also becoming apparent that carcinomas themselves may be viewed as "organ-like" populations, in which the processes of replication and differentiation are malfunctioning, producing incompletely or imperfectly differentiated cells that are aberrant but recognizable [12]. This concept, that cancerous growth recapitulates normal proliferative processes albeit in very dysfunctional ways, has tremendous implications for cancer therapy. For instance, most traditional cancer therapy targets the most rapidly growing (cancer) cells, resulting in impressive but frequently temporary clinical remissions. This likely occurs because the rapidly proliferating cancer transit-amplifying cells are successfully eliminated, while the slow-cycling cancer stem cells (CSCs) survive and reproduce a new round of progenitor offspring once therapy is completed. An increased understanding of the mechanisms of tumor replication in terms of CSC involvement will lead to more effective therapies. This article will review our current understanding of the development and biology of CSCs. We will re-interpret many well-known concepts in the field of cancer, including tumor clonality and heterogeneity, tumor initiation, pretumor progression, premalignant patches and

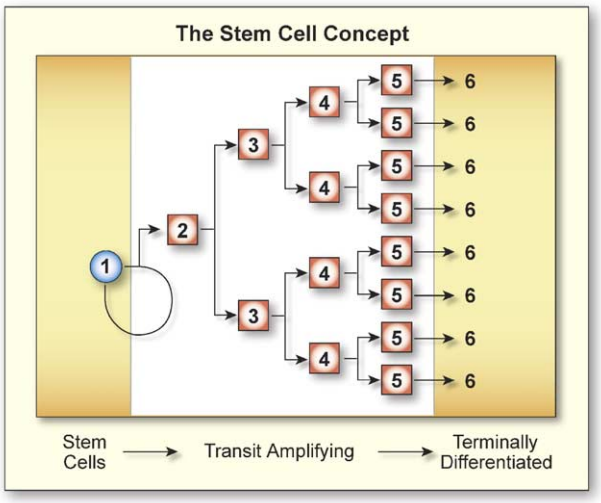

Fig. 1. The proliferative hierarchy in epithelia: the stem cell concept. The ultimate progenitor cells are termed *stem cells*. They are slow-cycling, long-lived, phenotypically undifferentiated, reside in specialized microenvironments, and constitute only a small percentage of the total epithelial population. Stem cell division produces *transit-amplifying* or *committed progenitor cells*, which cycle rapidly and produce a clonal expansion of the offspring arising from an initial stem cell division. These cells eventually become the mature *terminally differentiated cells* that constitute the bulk of a given epithelial population.

fields, field cancerization, tumor progression and cancer therapy in the newer context of stem cells and their niches. Wherever possible, recent reviews rather than original studies will be referenced.

2. What is a cancer stem cell?

2.1. Stem cells

Organs are composed of collections of differentiated cells that perform discrete functions. An underlying homeostatic system exists to replace senescent differentiated cells and tissue loss following injury. This hierarchical system typically involves several stages of cells that have decreasing reproductive capacity and simultaneous increasing commitment to differentiation (Fig. 1). The most primordial cell in the hierarchy, the *stem cell*, has the ability to reproduce for the life of the organ. It is typically undifferentiated, divides infrequently, and often resides in a specialized physical locale termed a "niche." Following division, a stem cell will give rise to, in average, one daughter stem cell that will remain in the stem cell niche and another, variously termed a *transit-amplifying cell* in most epithelial studies or a *multipotent progenitor* or *committed progenitor* in hematopoietic terminology. These rapidly proliferating cells undergo further reproductive divisions, and their offspring both expand the population of cells arising from the initial stem cell mitosis and progressively commit irreversibly to differentiation along one or several lineages.

Seven common and distinguishing features of stem cells have been described: (i) stem cells comprise a small subpopulation of a given tissue, (ii) stem cells are ultrastructurally unspecialized, with a large nuclear-to-cytoplasmic ratio and few organelles, (iii) stem cells can be pluripotent, (iv) stem cells are slow-cycling, but may be induced to proliferate more rapidly in response to certain stimuli, (v) stem cells have a proliferative reserve that exceeds an individual's lifetime, (vi) because stem cells cycle slowly, and represent only a small percentage of a cellular population, an intermediate group of more rapidly proliferating transient amplifying cells exists, that form clonal expansions resulting in the final, differentiated cell population and (vii) the microenvironment of a stem cell plays a critical role in its homeostasis and in the differentiation of its progeny [13].

Stem cell function involves self-replication (maintenance of the stem cell population), as well as production of offspring to maintain all lineages within the tissue during times of normal homeostasis as well as injury (e.g., wounding). Regulatory aspects of stem cell behavior may be *intrinsic* (within the programming of the stem cell itself), *extrinsic* (a response to external stimuli generated by the niche or imported soluble factors [14]) or, most likely, both. They include maintenance of the stem cell in a quiescent, infrequently cycling, undifferentiated state, decisions to replicate and in what fashion (*symmetrically*, producing two identical stem cells, or *asymmetrically*, producing one daughter stem cell and one daughter cell committed to differentiation: Fig. 2), and decisions to differentiate and along what lineage. Invariant, asymmetric division is common in unicellular organisms and invertebrates. A structured three-dimensional niche (a so-called *lineage* niche [15]) is typically required to partition the two different offspring. For example, in the *Drosophila* ovary, the daughter stem cell remains associated with somatic ("cap") cells of the basal terminal filament, while the committed daughter cell is displaced from the niche and differentiates into a mature egg [16]. Symmetric division appears to be more common in mammals, with extrinsic stimuli regulating the final size of stem and committed progenitor populations (Figs. 2, 3) [14]. For recent reviews of stem cells, see [3,7–11,15,17–23]. For brief historical highlights of our understanding of stem cell concepts, see Tables 1 and 2.

2.2. Cancer stem cells

As noted above, cancer growth can frequently be viewed as an aberrant version of normal tissue homeostasis. Although the end product is not a normal differentiated cell, owing to the multiple genetic mutations present and the frequently altered stromal microenvironment, nevertheless cancerous growth appears in many instances to recapitulate the stem cell → committed progenitor → differentiated cell procession that occurs during normal tissue reproduction.

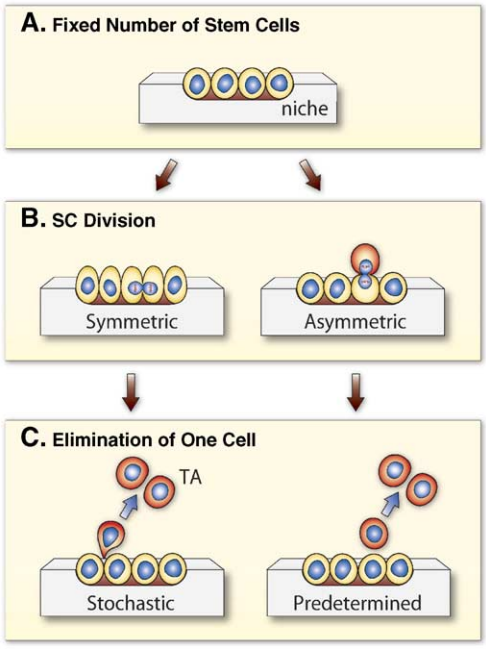

Fig. 2. Mode of division and the role of the niche in determining stem cell pool size. An important and unresolved issue is the factor(s) that determine stem cell pool size. We hypothesize that the niche is a predominant factor in regulating this attribute. Diagram A shows a hypothetical niche that can accommodate only four epithelial stem cells, which can divide in two modes. (B and C, left side) *Stochastic Model:* Mammalian stem cells can divide symmetrically [14] yielding two identical daughter cells resulting in a total of five identical stem cells in a space that can accommodate only four cells (B, left). Crowding leads to exit, randomly, of one of the stem cells: the cell that has departed loses its "stemness" and becomes a TA cell. (B and C, right side) *Predetermined Model:* Some recent data suggest that mammalian stem cells, like those of invertebrates, can undergo asymmetric cell division [102,103]. One daughter cell (the one still attached to the basement membrane in Diagram B, right side) retains the original DNA of the dividing stem cell and remains in the niche, while the other (the upper cell, marked in orange) is destined to leave the niche and become a TA cell. Such asymmetric division typically involves a highly structured niche setting in which the initial stem cell, prior to mitosis, is precisely oriented, and each daughter cell, following mitosis, is appropriately segregated [14]. If correct, this mode of stem cell replication has implications for cancer development [107] because it implies a natural protective mechanism: during mitosis the stem cell DNA remains a pristine template, avoiding the possibility of DNA replication errors.

Two conceptual models of tumor growth have been proposed [24,25]. In the older, *stochastic model*, all cells in the tumor have a high proliferative capacity. A growth fraction of <1 still occurs due to individual cell loss and non-reproduction, the result of constraints of the micro-environment such as focal lack of nutrients or oxygen, and age. However, in many cancers a *stem cell model* of tumor reproduction probably takes place. As in normal tissues, only a small percentage of the tumor population (i.e., the CSCs) maintain the capacity for long-term proliferation, while most cells proceed forward in a process of aberrant terminal differentiation. Variations in tumor growth rates may be the result of perturbations in the normal homeostatic mechanisms that regulate stem cell and TA cell reproduction (Fig. 3) or reflect alterations in the functioning of the niche-like CSC microenvironment (Fig. 4). CSCs arise from normal stem cells over time, in a process that parallels, and in fact underlies, the slow and multi-step development of cancer from normal tissues. This process will be discussed in detail in this paper. Differentiation of CSCs from normal stem cells within a given tissue is discussed in Section 5. For other recent reviews of CSCs, see [1,22,23,25–33]. For historical highlights of our understanding of CSCs, see Table 3.

3. Epithelial cancer stem cell characteristics

Several general CSC features have emerged from studies in the hematopoietic system and a number of epithelial types. They are described in the following sections.

3.1. Tumors frequently originate from the transformation of normal stem cells

A central tenet of cancer theory is that most cancers are clonal in origin [34–36]. It has been shown that cultured rodent cells require the introduction of two significant mutagenic events in genes critical to normal tissue homeostasis to become neoplastic [37,38], while kinetic analyses [37,38] as well as experiments with cultured human cells [39] indicate that four to seven such events are required for the creation of human cancers. Within these events, it has been suggested that six essential developments in cell physiology must occur: (i) self-sufficiency in growth signals, (ii) insensitivity to growth-inhibitory (antigrowth) signals, (iii) evasion of programmed cell death (apoptosis), (iv) limitless replicative potential, (v) sustained angiogenesis, (vi) tissue invasion and metastasis, and the development of a 7th characteristic that drives the first six: genome instability [37]. The somatic theory of cancer states that acquisition of these mutational changes occurs in a multi-step process termed *tumor progression* [40,41]. This pathway begins phenotypically with the earliest changes of cellular dysplasia or adenoma/papilloma formation. It then proceeds, typically although not always, through the pathophysiological stages of in situ disease, invasive cancer and finally, metastatic spread.

Multiple lines of clinical and experimental evidence suggest that stem cells are the target cell population in an organ in which mutations accumulate. (i) A curious phenomenon is that central corneal epithelium rarely, if ever, gives rise to carcinomas. The only carcinomas associated with cornea epithelium occur in the peripheral corneal in the limbal zone [42–44]. This region has been shown to be the site of corneal epithelial stem cells [4,45], thus providing perhaps thus far the most compelling evidence that, at least in corneal epithelium, carcinomas are stem-cell derived. (ii) The average epidermal cell turnover time has been calculated to be approximately 1 week in mice [46] and 6 weeks in humans

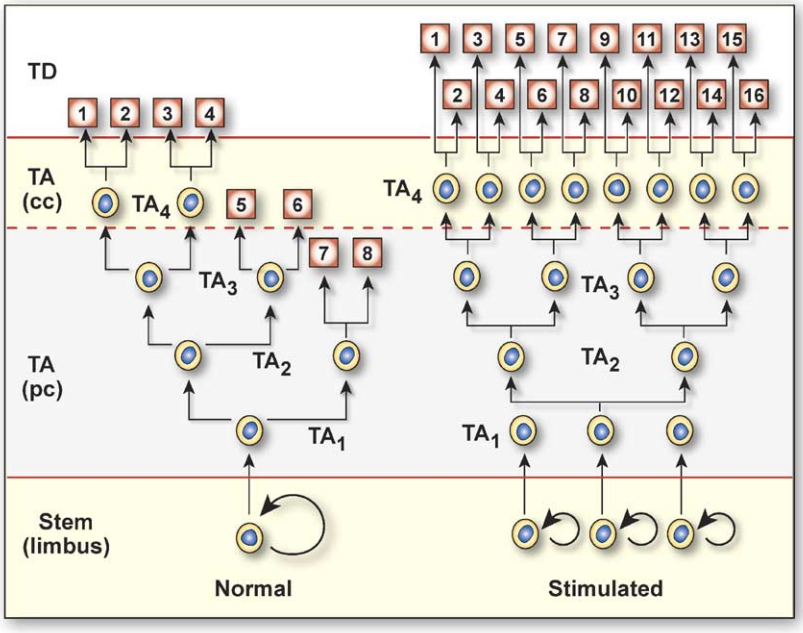

Fig. 3. Proliferative strategies during epithelial repair. Using corneal epithelial replication as a paradigm, this schematic summarizes the ways in which epithelial tissues can meet proliferative demands during, e.g., wound healing. In the normal situation (left), stem cells (S) located in the limbus [45] cycle infrequently with a relatively long cell cycle time (large curved arrow; [106]). Upon division, stem cells give rise to regularly cycling TA cells (vertical arrows) located in the peripheral (pc) and central (cc) corneal epithelium. Young TA cells (TA1) with multiple division capacity (shown here, for illustration purposes, with a maximum potential of three divisions) are preferentially located in the peripheral cornea, whereas the more mature TA cells (TA4) having little proliferative reserve reside in the central cornea and may divide only once prior to becoming terminally differentiated (TD; squares). Our data [381] suggest that under normal circumstances not every TA cell utilizes its full capacity to divide, represented by those TA cells with residual proliferative potential that nevertheless give rise to TD cells 5–8. The decision to become a terminally differentiated cell is stochastically determined. Upon stimulation, a self-renewing epithelial tissue can adopt three strategies to expand its cell population. It may recruit more stem cells to divide with a more rapid cell cycle time (smaller curved arrows) producing more TA cells. It may induce the young TA cells (TA2, TA3) in the peripheral cornea to more fully exercise their replicative potential thereby yielding more TA cells (i.e., a larger number of TA3 and TA4 cells than normal). Finally, it may increase the efficiency of TA cell replication by shortening the cell cycle time (shorter vertical arrows). Taken together, these three strategies result in the production of a significantly larger number of post-mitotic terminally differentiated cells than in normal epithelium. This model depicts an ideal case where all TA cells exercise their full potential and thus 16 terminally differentiated cells (instead of 8) are generated per stem cell division. Variations of these three strategies may occur in the growth-stimulatory cancer stem cell microenvironment.

[47]. This implies that only stem cells are present in the skin for prolonged periods of time, and thus capable of acquiring the multiple neoplastic changes over time that result in cancer. (iii) In two-stage mouse carcinogenesis studies, increasing the time interval between 7,12-dimethylbenz(a)anthracene (DMBA) initiation and subsequent promotion with either croton oil or 12-*O*-tetradecanoyl-phorbol-13-acetate to 40–63 weeks results in papilloma formation identical to that obtained when promotion is begun only 1–4 weeks after initiation [48–51]. DMBA forms covalent adducts in the DNA of target cells, and produces A→T transversions at the second nucleotide of codon 61 of the Harvey ras gene in essentially all papillomas and carcinomas that develop [52]. This implies that phenotypically normal, initiated cells maintain the Harvey ras mutation for the year prior to promotion; such initiated cells are by definition stem cells. (iv) In clinical studies, women exposed to the atomic bomb blasts at Hiroshima and Nagasaki had an increased incidence of breast cancer, often occurring 30 years after the time of exposure. Mutations in their cancers were consistent with those known to be induced by radiation. Those with exposure during late adolescence, when mammary gland stem cell numbers are highest, had the greatest susceptibility to develop breast cancer [8]. A similar, long latency period exists for the development of nonmelanoma skin cancers

Table 1
Highlights in our understanding of stem cells

1961—Till and McCulloch provide the first experimental evidence to support the existence of multipotent hematopoietic stem cells. They show that a population of clonogenic bone marrow cells termed colony-forming units can regenerate myelo-erythroid colonies in the spleen of lethally irradiated hosts [361].
1962—Lajtha et al. propose the kinetic model of stem cell replacement in the hematopoietic system [362].
1975—Cairns proposes mechanisms that promote “error-free replication” during stem cell reproduction [2].
1978—Schofield proposes the concept of a specialized “niche” that houses hematopoietic stem cells [187].
1996—Osawa et al. define cell surface marker combinations that isolate nearly pure mouse hematopoietic stem cell populations, and then reconstitute a full hematopoietic lineage from a single long-term hematopoietic stem cell [363].
1996—Reynolds and Weiss and colleagues grow highly purified neural stem cells as anchorage-independent “neurospheres” in culture suspension [364].
2003—Dontu et al. utilize neurosphere culture techniques to isolate nearly pure mammary stem cells as “mammospheres”[8].

Table 2
Highlights in our understanding of keratinocyte stem cells

1974—Potten proposes the concept of the "epidermal proliferative unit"[365].
1981—Bickenbach identifies "label-retaining cells" as putative epithelial stem cells by their slow-cycling nature [104].
1982—Lavker and Sun identify possible epidermal stem cells at the base of rete ridges in palm skin [112].
1986—Schermer et al. identify corneal epithelial stem cells [45].
1987—Barrandon and Green identify three types of clones in in vitro keratinocyte culture, termed holoclones, paraclones and mereclones, with differing proliferative potentials [366].
1989—Cotsarelis et al. identify label-retaining cells in the corneal limbus [106].
1990—Cotsarelis et al. identify label-retaining cells in the bulge of hair follicles [113].
1993—Jones and Watt enriched putative epidermal stem cells based on their expression of beta 1 integrin and rapid adherence to type IV collagen [367].
2000—Taylor et al. show that follicular stem cells are capable of forming not only elements of the hair follicle but also interfollicular epidermis [310].
2001—Oshima et al. document the multipotent nature of follicular stem cells [312].
2002—Liang and Bickenbach show that when purified epidermal stem cells are injected into mouse blastocysts, cells of epithelial, mesenchymal and neural crest lineage develop, indicating a plasticity similar to embryonic stem cells [258].
2004—Tumbar et al. [368] and Morris et al. [369] define the transcriptional profile of epithelial stem cells using different purification techniques (label-retaining cells versus those expressing keratin 15).

(NMSCs) in the skin following exposure to therapeutic radiation [53]. This therapy was used for many benign skin conditions, such as acne and tinea capitis ("ringworm") in the 1950s and 1960s, before it was known to induce skin cancer formation. (v) Epidemiologic studies find a correlation between episodes of intermittent overexposure to sun (e.g., sunburn) in childhood and subsequent basal cell carcinoma and melanoma formation in adulthood [54]. The average age of NMSC formation is 70 [55], yet epidemiologic data indicate that the majority of sun exposure occurs prior to the age of 18. This implies a lag time of decades between carcinogenic exposure and the subsequent phenotypic expression of cancer [56]. (vi) Only a tiny fraction of acute myeloid leukemia (AML) cells (0.02–1%, depending on the patient) are capable of transferring the disease when transplanted into immunodeficient (nonobese diabetic/severe combined immunodeficient or NOD/SCID) mice [57,58]. This population phenotypically resembles normal hematopoietic stem cells (HSCs) in having the [Thy1$^-$, CD34$^+$, CD38$^-$] phenotype, suggesting that they function as "leukemic stem cells." (vii) In patients with AML, the most common chromosomal abnormality involves an 8:21 translocation that produces AML-ETO chimeric transcripts. In patients in remission, these transcripts are identified in a variety of normal hematopoietic cells, in various stages of differentiation. This suggests that the 8;21 translocation occurred initially in a hematopoietic stem cell (HSC), that subsequently underwent additional mutations to produce a malignancy [59]. (viii) Similarly, 95% of chronic myeloid leukemia (CML) patients possess the Philadelphia chromosome, which serves as a cytogenetic hallmark of the disease. This fusion protein is found in myeloid, erythroid, megakaryocytic and B lymphoid cells in the majority of CML patients, suggesting that the original translocation takes place in a HSC [60]. (ix) Stem cells also appear to be the source of malignant transformation in Philadelphia chromosome-positive acute lymphocytic leukemia [61]. (x) In examining the origins of breast cancer, cells from nine human breast patients were separated into different fractions on the basis of cell surface marker expression. As in AML, only a small and highly specific population was able to induce tumor formation when transplanted into NOD/SCID mice. As few as 100 of these cells could consistently form tumors. They had a definable phenotype [CD44$^+$, CD24$^{-/low}$, Lineage$^-$], they behaved like stem cells in that they could be serially passaged, and they gave rise to diverse populations of cells with multiple, differentiated phenotypes [30]. (xi) Similar results have been obtained in pediatric brain tumors, where a

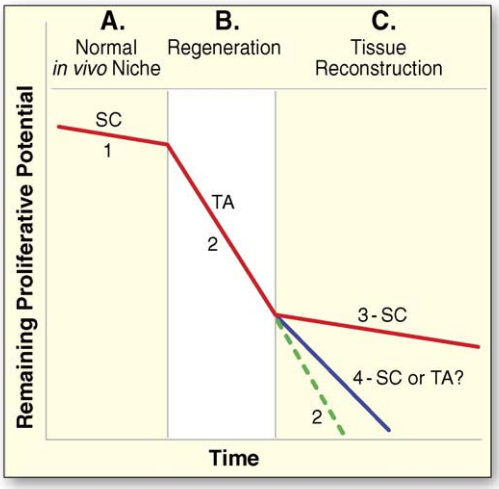

Fig. 4. Role of the niche in maintaining/preserving stem cell proliferative potential. (Line 1) The niche microenvironment is thought to play an important role in maintaining the slow-cycling and relatively undifferentiated stem cell state. In this setting, stem cells waste little of their proliferative potential. (Line 2) Loss of the normal niche due to, e.g., tissue damage or the transfer of the stem cells to a tissue culture environment, can cause the stem cells to undergo rapid proliferation, thus behaving like TA cells, with a rapid decline of their remaining proliferative potential. (Line 3) Such TA cells can potentially be converted back to slow-cycling stem cells if they are replaced into another normal stem cell niche, such as when wound healing is completed or following the in vivo transplantation of cultured cells. Stem cell properties are restored, although these cells will have a lower ultimate proliferative potential than that of the original stem cell. (Line 4) If replaced into an inadequate niche setting, stem cell properties can be only partially restored and the cell behaves more like a TA cell. According to this hypothesis, the boundary between stem and TA cells is not absolute or determined by any intrinsic differences between the two cell types. Rather, stem and TA cells represent different functional states as defined by their environment [269]. Such a blurring could also occur as a normal stem cell becomes a cancer stem cell, and its normal niche is replaced by the altered cancer stem cell microenvironment.

Table 3
Highlights in our understanding of cancer stem cells

1875—Cohnheim proposes that misplacement of stem cells (*Muskelkeimzellen*) during embryonic development can lead, in later life, to the development of tumors [370].

1921—Rotter postulates that sex cells might lodge outside of glands and serve as an origin of tumors, although such embryonal nests are never found [371].

1956—Makino examines chromosomes from ascites tumor cells in rats, and finds that the highly characteristic chromosomal abnormalities suggest clonal origins [372].

1963–1967—Bruce and van der Gaag [373] and Wodinsky et al. [374] show that only 1–3% of transplanted leukemic cells are able to form spleen colonies in vivo.

1964—Kleinsmith and Pierce show that a single embryonal carcinoma cell can produce a heterogenous assortment of offspring containing up to 14 different tissue types, implying that teratocarcinomas arise from a pluripotent malignant stem cell [375].

1971—Park and coworkers, using in vitro colony forming assays, calculate that 0.7–1.2% of leukemic cells are CSCs [376].

1977—Cairns postulates several mechanisms that may naturally retard the development of mutations in tissue stem cells, and suggests that development of clones containing a "primer mutation" may facilitate the mutagenic process [2].

1994—Sell and Pierce propose that blocked differentiation ("maturation arrest") underlies cancer stem cell proliferation and tumor growth [377].

1997—Blair et al. [57] and Bonnet and Dick [58] show that only a small % of acute myeloid leukemia cells (0.02–1%), which phenotypically resemble hematopoietic stem cells can transfer disease when transplanted into rodent hosts.

2003—Al-Hajj et al. show that in human breast cancers, only a small, specific population of cells are able to induce tumor formation after transplantation into NOD/SCID mice [30].

2003—Singh et al. show that in pediatric brain tumors, a small subset of cells expressing neural stem cell marker CD133 account for almost all in vitro proliferative activity [62]. Similar results are obtained by Hemmati et al. [64] who generate neurosphere-like growths of pediatric brain tumor cells in cultures.

small subset of cells expressing the neural stem cell marker CD133 have been shown to account for almost all in vitro proliferative activity. When cultured, they give rise to a diversity of cell phenotypes resembling those of the original tumor, and injection of small numbers of such cells into NOD/SCID mice produces tumors [62,63]. (xii) In another set of experiments, neurosphere-like growths of pediatric brain tumor cells were generated in culture. They expressed many genes characteristic of neural stem cells, and when transplanted into rat brains they could be serially passaged and produce multiple differentiated neural elements [64]. (xiii) In long-established C6 glioma cell cultures, only the small percentage (0.4%) of the population containing side population markers (associated with stem cells) could reproduce both side population and non-side population cells, form neurospheres, neurons and glial cells, and produce tumors with high efficiency when injected into nude mice [65]. (xiv) In the lung, a stem cell paradigm exists for tissue homeostasis [9]. Some light and electron microscopic findings suggest that type II pneumocyte stem cells are precursors to both adenocarcinomas and squamous cell carcinomas. (xv) Finally, in a recent study, cultures of metastatic melanoma cells were examined for the presence of CSCs. Small ovoid cells were observed and clonally purified. These cells had the capacity to self-propagate, and they differentiated into a secondary cell population with an increased proliferative rate, increased TRP-1 expression and increased numbers and maturation of melanosomes, i.e., they behaved like stem cells [66]. Collectively, these data suggest that mutations leading to cancer formation in humans accumulate over a period of decades, and imply that stem cells must be the targets of carcinogenic exposure.

3.2. In some instances, cancer can also develop from a restricted progenitor cell

Two clinical observations and a body of in vitro experimental evidence suggest that cancer might also arise from, or at least involve, more committed precursor cells. It is also possible that the replacement of a normal stem cell niche by a CSC microenvironment may blur the distinctions between stem cells and committed precursors (Fig. 4). However, given the above-noted prolonged timeframe of in vivo cancer development in humans, what the data may reflect is that, although early mutations occur in stem cells, the final transforming events can take place, in some instances, in downstream, early progenitors. (i) In acute promyelocytic leukemia (APML), the most differentiated subset of AML, the APML-associated fusion gene PML/retinoic acid receptor-α is present in cells with the [$CD34^-$, $CD38^+$] committed myeloid progenitor phenotype, but not in cells with the [$CD34^+$, $CD38^-$] HSC phenotype [24,67]. This suggests that the final transformation events in APML specifically occur during a more differentiated stage. (ii) As noted above, leukemic stem cells have a surface marker phenotype that is similar to normal HSCs [58]. However, it has been noted that some differences in Thy-1 and IL-3 receptor-α expression exist [68,69], suggesting the possibility that final malignant conversion takes place in a more differentiated precursor. (iii) A number of in vitro experiments can produce leukemia from committed precursor cells. Use of the hMRP8 promoter to target oncogenes specifically to myeloid committed progenitors but not HSCs in transgenic mice can produce AML-like, CML-like and APML-like disease [22,70], and most normal human cells can be made tumorigenic by forced expression of a defined set of viral and cellular proteins [39,71]. (iv) Similar work has been performed in breast tissue, resulting in transformation of committed progenitor-like cells termed "pregnancy-induced mammary epithelial cells" [72]. (v) Transduction of a constitutively active epidermal growth factor receptor into either neural stem cells or more-differentiated astrocytes from $Ink4a/Arf^{-/-}$ mice produces a similar, high-grade glioma phenotype in both cases [73]. (vi) Cultured epidermal cells with limited lifespans (termed *paraclones*) can acquire an infinite lifespan by the acquisition of viral oncogenes [74]. Transgenic expression of c-*myc* in the suprabasal compartment of mouse skin via

an involucrin promoter stimulates proliferation and papilloma formation [34]. Double transgenic mice, that express both TGF-α and v-fos in suprabasal epidermal cells via a keratin 1 promoter, develop papillomas that rapidly convert to carcinomas, following mutation of the Ha-ras gene by promotion with TPA [75]. (vii) One uncommon form of skin cancer with a clinical history that suggests formation from a committed progenitor cell type is the *keratoacanthoma* [76]. Long considered as a benign entity because of its frequent tendency to spontaneously resolve over 3–6 months following rapid formation, the keratoacanthoma is now more commonly conceived of as a subtype of SCC because of occasionally documented metastases. The keratoacanthoma's life cycle of rapid growth followed by slow regression simulates the proliferative life cycle of a TA cell located in the hair bulb through anagen, catagen and telogen, suggesting an origin from it. However, again, the relevance of these models to the actual, prolonged in vivo process of carcinogenesis in humans is unclear.

3.3. Cancer stem cell development begins with the acquisition of mutations in normal stem cells—a process termed "pretumor progression"

Most current systems to grade and stage cancer are based on histopathologic examination of cancerous tissues and cells. The earliest steps in cancer development and progression reflect phenotypic changes under the microscope; pathologists say that cancer "begins" when such morphological changes can first be noted. Typical histopathologic changes that occur during cancer development and subsequent progression include mild, moderate and severe dysplasia, carcinoma in situ and then invasive carcinoma that may have well-differentiated, poorly-differentiated or undifferentiated features. However, in the molecular age, it is becoming clear that such histologic staging reflects a historical but insensitive technology that is capable of identifying only phenotypic but not genotypic changes in transforming cells. Mutations almost certainly occur in stem cells before any phenotypic changes become evident. Clonal expansion of mutation-harboring stem cells within a niche, termed *pretumor progression* [77], and then beyond the confines of an individual niche into microscopic-sized *patches* and still-larger *fields* [78], both involve and potentiate the acquisition of additional mutations by the expanding clonal population, and this drives the process of early carcinogenic progression forward.

The pretumor progression theory of cancer [77] suggests that cellular acquisition of somatic mutations frequently occurs before phenotypic changes are visible, that the process essentially begins at birth, and that it frequently involves clonal evolution of stem cells in niches, in a manner analogous to the clonal evolution of cancer stem cells that occurs during classic tumor progression, after the process becomes "visible". A number of diverse lines of data support the first two tenets: (i) In two-stage carcinogenesis studies, as noted earlier, initiation of animals with DMBA produces A→T transversions in the Harvey ras gene that persist for at least 40–63 weeks in phenotypically normal cells [48–52]. (ii) In genetic cancer syndromes involving tumor suppressor genes (e.g., retinoblastoma, familial adenomatous polyposis, nevoid basal cell carcinoma syndrome) [79,80] phenotypically normal individuals harbor heterozygous genetic changes in all of their somatic cells, including stem cells, at birth. (iii) Studies of childhood leukemia in identical twins have documented a common clonal origin in some instances, indicating that initiation occurred prenatally [81], and molecular examination of the cord blood of normal children at birth has revealed the presence of common leukemia fusion genes [82]. These studies indicate that acquisition of mutations potentially involved in cancer development can actually occur *before* birth. (iv) Similarly, intrauterine exposure to diethylstilbestrol is associated with the development of vaginal adenocarcinoma [83] and an increased risk of breast cancer [84] in adult women and possibly testicular cancer in men [85]. In addition, elevated birth weight in women is associated with an increased risk of developing breast cancer as an adult; the mechanism may involve elevated levels of hormones and insulin-like growth factors, which increase the absolute number of mammary stem cells present that are then at risk for undergoing subsequent malignant transformation [86–88]. (v) Genetic changes have been documented in phenotypically normal tissues surrounding many epithelial cancers [78] including those from the head and neck region [89–91], bladder [92,93], colon [94], breast [92,95] and skin [96]. (vi) Some invasive cancers, such as nodular melanoma and some cases of colorectal cancer [97], arise relatively rapidly and without evidence of progression through common precursor stages. This suggests that in these instances, an appropriate additional mutational event can produce the full cancer phenotype in an already mutated but phenotypically still apparently normal stem cell. The third tenet – that clonal evolution occurs in stem cell niches – has only recently been addressed. DNA methylation work on human colon crypts [98,99] indicates that in normal niches containing multiple stem cells, random stem cell turnover occurs, probably through symmetric division of a stem cell into two daughters destined for differentiation, resulting in its replacement by other stem cells. Thus, over time, a clonal succession occurs. In the human colon crypt, a succession cycle appears to occur approximately every 8 years [99]. Regarding the process of carcinogenesis, any genetic alterations in a stem cell that increase its "fitness" within the niche can lead to clonal expansion. Many genetic alterations are probably neutral or detrimental to cycling, and will either be eliminated or passively rise to clonal dominance during a niche succession cycle. However, clonal expansion at the stem cell niche level, like clonal expansion in phenotypically cancerous cells, increases the likelihood that additional mutations may be acquired by one of the clonal cells (Fig. 5). Recent DNA methylation studies

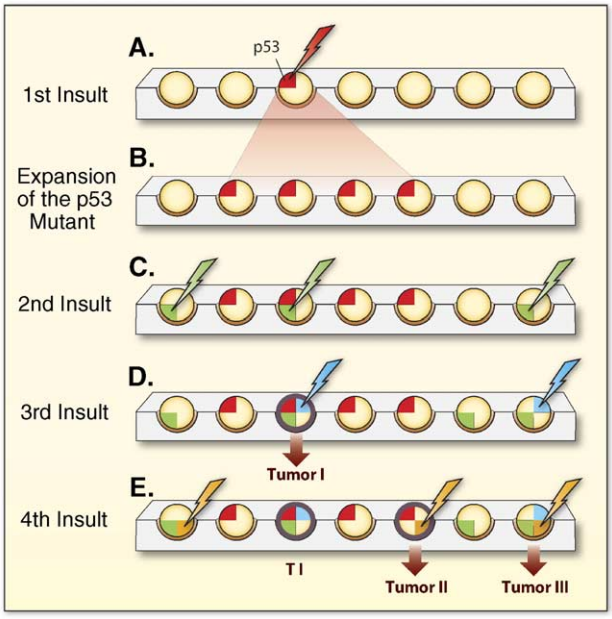

Fig. 5. The role of stem cells in the clonality of pretumor progression. In epithelia exposed to a carcinogen, cancers can form at multiple foci that may be both unrelated or of a common clonal origin. Genetic markers are useful in determining the mono- or oligoclonality of multiple tumors. (A) A hypothetical epidermis contains seven stem cells, each contacting a separate niche. Following ultraviolet irradiation a particular gene (colored red; the p53 gene in this hypothetical example) of cell 3 is mutated, conferring a growth advantage relative to its neighbors. (B) This cell replicates more quickly, like a TA cell, and its progeny either displace or replace neighboring stem cells 2, 3, 4 and 5 [114]. (C) An additional environmental insult produces a further mutation in another gene (green) in cell 3 as well as in cells 1 and 7. (D) Another environmental insult produces an additional mutation of another gene (blue). The combined oncogenic changes in cell 3 are now sufficient to form a cancer (Tumor I). (E) With another mutation (yellow), cells 5 and 7, that have accumulated different combinations of oncogenic mutations, form cancers as well (Tumors II and III). This hypothetical case involves the formation of three separate cancer foci (I, II and III), in which two are clonally related (I and II) while the third (III) is derived independently. Whether once can reconstruct this lineage depends on which oncogenic events (markers) are analyzed. Analysis of the p53 (red) gene may reveal the relatedness of Tumors I and II, while analysis of the green gene may lead to a different conclusion.

of individuals with familial adenomatous polyposis [100] and hereditary nonpolyposis colorectal cancer [101], who carry heterozygous mutations in adenomatous polyposis coli and DNA mismatch repair genes, respectively, support the notion that pretumor progression occurs through stem cell niche succession.

Finally, it has recently been proposed [102,103] (Fig. 2) that mouse intestinal stem cells arrange their sister chromatids at mitosis so that the same template DNA strands stay together through successive divisions, i.e., a rigid form of asymmetric division occurs in which the original DNA strand stays with the offspring cell that maintains stem cell status. It is postulated that this is also the mechanism underlying "label-retaining cells" seen in experiments in skin [104,105], cornea [106] and prostate [10]. As noted earlier, this type of division presumably involves a highly structured niche setting in which the stem cell, prior to mitosis, is precisely oriented to yield a stem cell and a transit-amplifying (TA) cell (Fig. 2). It has also been suggested that DNA excision repair within the stem cells is inhibited, in order to prevent sister chromatid exchanges that could impair their genetic purity [107]. In this scenario, any significant damage to a stem cell results, not in repair, but in apoptosis. If correct, this method of stem cell replication has implications for cancer development [107] because it implies that an initial mutagenic event must involve the death of a stem cell that is then replaced by a daughter cell that harbors a mutation. Whether this conception is correct remains to be seen.

3.4. Pretumor progression of stem cells gives rise to clonal expansions termed "patches" and "fields" that are responsible for the field cancerization effect seen in many epithelia

Following clonal stem cell expansion (pretumor progression) within a niche, the next step in the very early carcinogenic process is the extension of this process beyond the confines of the individual niche (Fig. 5). Such clonal expansion has been well-documented in broad regions of epithelia exposed to carcinogens, often adjacent to sites of documented cancer [78]. Small-sized clonal expansions, termed *patches*, are present in phenotypically normal epithelia, and have been observed in several organs, including the head and neck region [108], lung [109], breast [95], gastrointestinal tract [110] and skin [96]. Larger regions of apparently contiguous clonal outgrowths, termed *fields*, may be quite large and can manifest histologic features of atypia, such as hyperplasia or dysplasia. One epithelium in which patches have been well studied is the skin. There are frequent clonal populations of p53-mutated keratinocytes in histologically normal human skin. They have been termed "p53 patches" [96,111]. These p53 patches are more frequent and larger in sun-exposed skin [111], and most of the mutations involve C→T or CC→TT substitutions, indicating they are produced by ultraviolet light [111]. Thus, ultraviolet light appears to be the causative carcinogen underlying epidermal patch formation. On examination in epidermal whole mounts, the p53 patches arise from the dermal–epidermal junction as well as hair follicles. Two thirds of the smaller interfollicular clones are shaped like inverted cones, with their apex near the dermal–epidermal junction, presumably originating from a basally located, transformed interfollicular epidermal stem cell [112]. Follicular clones encircle the follicle, where follicular stem cells are located in the region of the bulge [113]. The shapes of these patches suggests that they are clonal outgrowths arising from stem cells. However, they are 60–3000 cells in size [111], which is much larger than the number of stem cells residing in a typical epidermal niche. A majority of the cells in these p53 patches are

resistant to ultraviolet light-induced apoptotic death [111]. Thus, it is theorized that the patches develop through a clonal expansion of stem cells that are resistant to ultraviolet-induced apoptotic death: after surviving ultraviolet irradiation, the mutant clonal stem cell population expands into the vacated niches of their now-deceased normal neighbors (Figs. 5A and B) [111,114,115]. Indeed, ultraviolet-induced apoptosis of stem cells originally occupying neighboring normal niches appears to be crucial to this early clonal expansion process [116]; in transgenic mice in which the anti-apoptotic protein survivin is expressed in the skin, ultraviolet-induced clonal expansion of mutant p53 patches is decreased [117].

Fields represent an even larger clonal expansion of genetically altered stem cells. These fields frequently [89,108,118], although not always [91,92,94,119], show histologic evidence of dysplasia. Some fields can be many centimeters in size [118,120,121], indicating significant migratory spread. They have been observed in many epithelia, including the head and neck region [89,108,122], lung [121], bladder [123], cervix [124], colon [94], breast [92] and Barrett's esophagus [118]. Although usually not referred to by the term "fields," regions of ulcerative colitis in the colon containing mutations [125,126] and actinic keratoses, which are dysplastic precursors of squamous cell carcinoma [127,128] that develop on sun-exposed regions of skin, constitute epithelial fields as well. It is hypothesized that clonal patches are a precursor of the larger clonal fields [78,108]. Evidence in support of this includes: patches and fields both contain mutations and develop in close proximity in regions that have been exposed to carcinogens; clonal patches usually do not show histologic evidence of dysplasia while fields frequently do; and clonal fields frequently show greater numbers of mutational events than patches [90,96,109,129,130]. However, it is also possible that patches represent a benign monoclonal phenomenon and not a necessary step in the process of cancer development. Evidence against patches being precursor lesions for fields or subsequent cancers includes: genetic studies of skin [96,129,131], head and neck epithelia [108] and lung [109], in which disparate mutations are noted in patches and immediately adjacent fields or cancers, suggesting a lack of common origin; prevalence assessment of epidermal p53 patches in human skin, in which patch density does not correlate with frequency of skin cancer formation [132]; and murine experiments, in which a large percentage of ultraviolet-induced p53 patches rapidly regress once the source of ultraviolet light exposure is removed [114,133]. One confounding factor may be that progression of patches to fields is simply an uncommon event. Keratinocyte p53-mutant patches, for example, are estimated to be 100,000 times more common in human skin than field-like actinic keratoses [96]. If only a tiny percentage of patches progress to become a field or a cancer, studies-to-date may simply have not observed this uncommon event. It is clear, however, that clonal fields are precursors of subsequent carcinoma development. Slaughter et al. first proposed the concept of *field cancerization* in 1953 [134] to explain the common development of second primary cancers in head and neck epithelia that had been previously exposed to carcinogens. In this classic sense, field cancerization refers to multiple, unrelated (non-clonal) carcinomas arising within common epithelia as a result of similar carcinogen exposure (Fig. 5). However, more recent experimental work has evolved the concept of clonal fields, as noted above, in which the acquisition of additional mutations by subclones within fields can lead to potentially multiple cancers that all have a common origin (Fig. 5) [89,135]. It actually appears that both oligoclonal [89] and monoclonal [89,108,124,135] cancer development occurs in regions of epithelia exposed to carcinogens [123,136]. In a majority of cases, common mutations are identified in multiple carcinomas arising in close proximity [137–139], or in clonal field cells and carcinomas arising within or adjacent to the field [89,90,92,108,124,140], indicating a common origin. However, genetically unrelated cancers [120,139] and cancers unrelated to a nearby field [89,109] also develop within carcinogen-exposed regions of epithelia; in this case, they are the product of a field cancerization effect, but they have not arisen from the same clonal precursor (Fig. 5).

3.5. Further progression of early cancer stem cells leads to the development of frank "cancer," and continued progression within new subclones results in the development of increasingly aggressive and heterogeneous cancer phenotypes

The development of frank carcinomas within clonal fields involves the acquisition of additional mutations, in addition to those already present within the field itself (Fig. 5) [89–91]. This indicates a process of continuing progression. *Tumor progression* refers to the acquisition over time, within an already-existing cancer, of increasingly malignant features [141]. Examples of features that increase the likelihood of metastasis [142] are the acquisition of an invasive phenotype, or those that enhance the rate of tumor growth, such as an increase in the growth fraction, i.e., the percentage of cells in the population that actively cycle. Tumor progression is thought to occur as the result of selective pressures that lead to the expansion of certain clonal subpopulations [143] and the diminution or loss of others. This process, along with the differentiation of cancer stem cells into (aberrant) differentiation pathways, results in tumor *heterogeneity* [1,144,145]. Specific models of genetic tumor progression, based on the multi-hit somatic theory of cancer, have been well-characterized in a number of malignancies, including colorectal cancer [40], head and neck cancer [135] and melanoma [41]. As in pretumor progression, to persist over the months-to-years in which the process takes place in vivo, frank tumor progression involves the cancer stem cells and their progeny.

3.6. Similar signaling pathways appear to regulate self-renewal in normal and cancer stem cells; hence mutations in pathways involved in embryologic development and stem cell homeostasis can result in cancer formation

Because of their important and relatively unique status, mutations in genetic pathways involved in stem cell regulation could play a role in cancer formation. Furthermore, because many of these pathways are also involved in embryologic development, germ line mutations in them that are not overwhelmingly lethal have the potential to underlie hereditary cancer syndromes. It is now clear that a number of genetic pathways involved in stem cell self-renewal are also involved in cancer development. Genetic pathways in this category include Wnt, Hedgehog, Notch, Myc and PTEN. We will focus on two of these pathways in this review, Wnt and Hedgehog.

3.6.1. The Wnt–β-catenin–Lef/Tcf pathway

The Wnt pathway is diagrammatically represented in Fig. 6. Wnt proteins are involved in organism patterning during development, as well as stem cell lineage determination and homeostasis in the adult. The Wnt pathway is implicated in the pathogenesis of a number of cancers, usually by activating mutations that result in a ligand-independent state of constitutive activity [33].

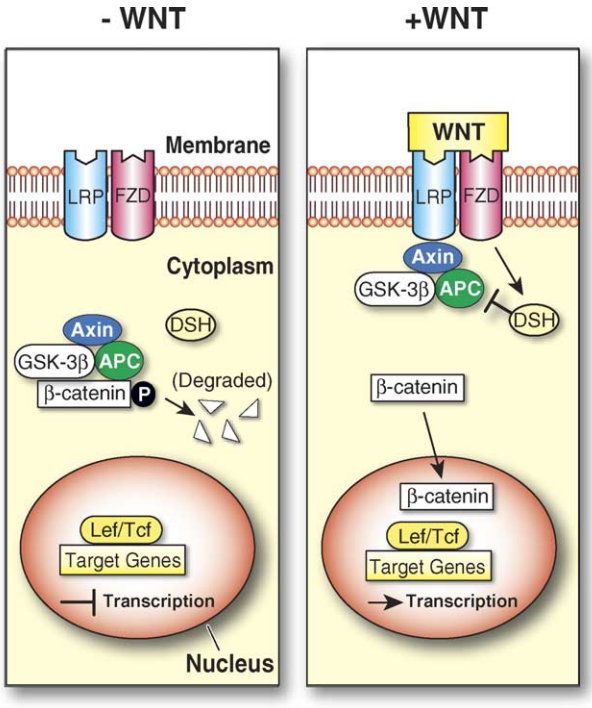

Fig. 6. The WNT–β-catenin–Lef/Tcf Pathway. The binding of a soluble WNT protein to Frizzled (FZD) and LDL-receptor-related protein 5/6 (LRP) transmembrane receptors leads to their binding to the intracytoplasmic proteins dishevelled (DSH) and axin, respectively [382,383]. These interactions disrupt the triple complex consisting of axin, glycogen synthase kinase-3β (GSK-3β) and adenomatosis polyposis coli (APC), which normally phosphorylates β-catenin leading to its degradation. Thus, WNT stabilizes β-catenin, allowing it to accumulate, migrate to the nucleus, and bind to lymphoid enhancer factor/T-cell factor (Lef/Tcf) receptors which activate target genes that promote growth and differentiation. A soluble inhibitor of WNT exists, named Dickkopf-1 [156].

Overexpression of the Wnt pathway stimulates stem cell proliferation in a number of organ systems. In *Drosophila*, the wingless gene (analogous to Wnt in mammals) is necessary for maintaining somatic ovarian stem cells. Downregulation of wingless results in rapid stem cell loss, while constitutive signaling causes overproliferation and abnormal differentiation of somatic follicle cells [146]. In the hematopoietic system, overexpression of β-catenin expands the pool of hematopoietic stem cells (HSCs); while inhibitors of the Wnt system lead to inhibited HSC growth [147]. Wnt signaling is required for self-renewal of gut stem cells. It is involved in intestinal embryogenesis and adult intestinal epithelial cell proliferation [7]. Mice with a targeted deletion of the Tcf-4 receptor lack a stem cell compartment in the small intestine. Transgenic mice expressing a stabilized (active) β-catenin show an expansion of the neural precursor cell population [148]. Wnt pathway molecules show a different expression in mammospheres (which are composed primarily of mammary stem cells) compared to differentiated mammary cells, suggesting involvement in the regulation of normal mammary stem cell function [8]. Finally, Wnt pathway stimulation within epidermal stem cells results in commitment along a follicular differentiation pathway [149]. In vitro studies indicate that Wnt system signaling maintains the hair-inducing activity of dermal papilla [150]. Overexpression of Lef-1 in transgenic mice leads to ectopic formation of abnormal hair follicles in interfollicular and oral epithelium [151,152], while genetic ablation of Lef-1 leads to arrested follicle development and hairlessness [153]. Similarly, transgenic expression of β-catenin in mice leads to ectopic hair formation [154], but when the β-catenin gene is ablated in mice [155], or when Wnt signaling is inhibited by ectopic expression of Dickkopf 1 [156], hair placode formation does not take place during embryogenesis. This suggests that Wnt-stimulated β-catenin activation of Lef-1/Tcf-1 signals epidermal stem cells to commit to follicular, rather than epidermal, differentiation [155].

Constitutive overexpression of the Wnt system is observed in a number of cancers [33,157]. In the hematopoietic system, β-catenin is involved in leukemia cell proliferation, adhesion and survival [158]. In the gastrointestinal system, mutations in the APC gene underlie the syndrome *familial adenomatous polyposis*, in which multiple adenomas and subsequent carcinomas develop with greatly increased frequency. The APC gene is mutated in 80% of sporadic colorectal cancers as well, in what is thought to be a very early, if not primary, step in colon carcinogenesis [7]. Wnt pathway dysfunction is an important component of prostatic tumorigenesis [159].

In the epidermis, constitutive overexpression of the Wnt pathway stimulates the development of follicular tumors, while constitutive underexpression leads to the production of sebaceous and interfollicular tumor types. Transgenic expression of stabilized β-catenin in mouse skin leads to development of several follicular tumors, including epidermal cysts, trichofolliculomas, and with more time, pilomatricomas [152,154]. Conversely, transgenic expression of an Lef-1 lacking a β-catenin binding site in mice leads to progressive hair loss and the development of epidermal cysts that appear to arise at the base of anagen hair follicles. After several months, several sebaceous and interfollicular epidermal tumors develop, including sebaceous adenoma, seboma, squamous papilloma and squamous cell carcinoma [160]. Finally, β-catenin is also mutated in sporadic medulloblastomas [161] and several other cancers [162]. Transgenic expression of Wnt-1 in mice produces adenocarcinomas [163], and human head and neck SCCs frequently overexpress the Wnt pathway [164]. In summary, the Wnt pathway plays active roles in embryogenesis, stem cell homeostasis and cancer formation in a number of organ systems.

3.6.2. The Hh–Ptch–Gli pathway

A schematic diagram of the Hedgehog (Hh) pathway is shown in Fig. 7. The Hh system is involved in embryonic patterning as well as stem cell proliferation, survival and growth. Constitutive overexpression of Hh is observed in a number of cancers.

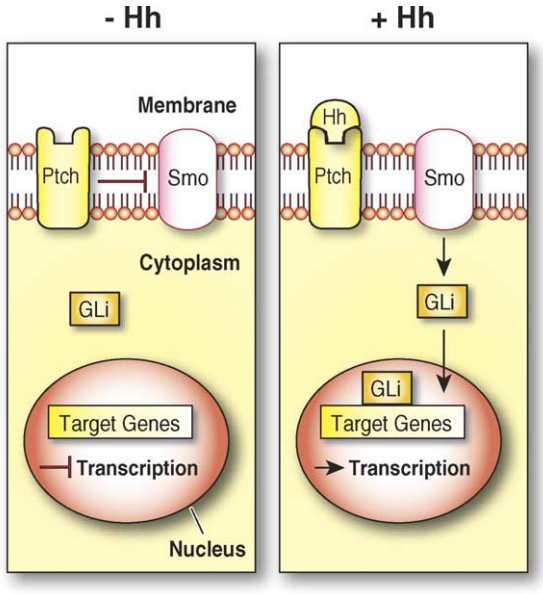

Fig. 7. The Hh–Ptch–Gli Pathway. Patch (Ptch) is a transmembrane protein that constitutively inhibits the functioning of another transmembrane protein, termed Smoothen (Smo). When the soluble protein Hedgehog (Hh) binds to Ptch, its repression of Smo is released, allowing a downstream cascade of events to occur. One family of downstream effector molecules in many instances is Gli [180].

The Hh pathway regulates stem cell turnover in several organ systems. In the *Drosophila* ovary, Hh acts as a somatic stem cell factor; in its absence, *Drosophila* somatic stem cells cannot proliferate, while excess Hh system signaling stimulates the production of somatic stem cells [165]. Sonic Hh (the main subtype of Hh found in mammals) induces proliferation of primitive human hematopoietic [166] and gut progenitor cells [167], and regulates neural [168,169] and airway [170] progenitor cell proliferation. In epidermis, stimulation of the Hh pathway promotes proliferation of committed follicular progenitors [149,171], but the system does not appear to be involved in interfollicular progenitor cell homeostasis. Several observations suggest this. (i) Hh pathway expression is largely restricted to the hair follicle, with little expression noted in interfollicular epidermis [171,172]. (ii) Hh pathway target genes are expressed in the skin only during the anagen portion of the hair cycle [173]. (iii) Ectopic expression of the Hh pathway in K14-sonic Hh transgenic mice leads to the development of BCC-like tumors in hair follicles but has little apparent effect on interfollicular epidermis [171]. (iv) During embryonic follicle development, sonic Hh transcripts are expressed in the epithelial cells of each newly formed, proliferating follicle, while Ptch mRNA is seen in each adjacent dermal condensate [171]. (v) During the adult anagen hair cycle, a similar "complementary" expression of Hh and Ptch is seen [174]. Thus, the HH system appears to be essential for both embryonic hair follicle development and the postnatal hair growth cycle [173,175,176].

Constitutive overexpression of the Hh pathway is observed in a number of cancers. Gain-of-function mutations underlie the *nevoid basal cell carcinoma syndrome* [79,177–179] in which affected individuals have an increased risk of developing medulloblastomas, primitive neuroectodermal tumors of the cerebellum, and basal cell carcinomas (BCCs) of the skin [180]. Ptch mutations are also found in some sporadic medulloblastomas, Ptch and Gli-1 gene overexpression are noted in many primary central nervous system tumors [180], and overexpression of the pathway is present in essentially all syndrome-related as well as sporadic BCCs [171–173,181–184]. Constitutively increased Hh system signaling also appears to be a necessary presence in a subset of small cell lung cancers [170] as well as in many digestive tract tumors, including those of the esophagus, stomach, biliary tract and pancreas [185,186]. Thus, the Hh pathway is involved in embryogenesis, stem cell homeostasis and cancer formation in a number of organ systems.

3.7. Stromal–epithelial interactions are important to both stem cell maintenance (the stem cell "niche") and cancer development

The concept of a hematopoietic stem cell "niche" was first put forth by Schofield in 1978 [187], and was recently termed a "hematon" in the mouse [188]. Although the

specifics appear to vary significantly in different tissues, as a concept the term "niche" refers to a three-dimensional microenvironment composed of mesenchymal cells and extracellular matrix molecules that support the presence and function of stem cells. They play an important role in regulating stem cell reproduction as well (Fig. 2). Stem cell niches have been studied in many organs, including bone marrow [188], brain [189,190], intestine [191], breast [192], cornea [4,193], kidney [194], epidermis [195], melanocytes [196,197] and dermis [198]. For recent, general reviews, see [14,15,199,200].

The somatic mutation theory of cancer has been the prevailing paradigm in cancer research for decades [201]. It states that the accumulation of multiple mutations, amplifications and/or deletions within a single cell in genes critical to normal tissue homeostasis is the essential process that leads to cancer formation [202]. However, recent attention has turned to the additional role of the microenvironment in which cells undergoing transformation exist in cancer formation. Just as the stem cell niche plays a critical role in the maintenance and functioning of normal stem cells, so the microenvironment of premalignant and malignant cells has been shown to have major effects on the development and progression of cancer (for recent reviews, see [12,202–209]). The effects of a microenvironment on cancer cells are most commonly epigenetic [210]; they produce potentially reversible alterations in cellular homeostasis that may involve modifications of chromatin [211] but do not involve changes in the actual DNA sequence of genes. It is becoming clear that these effects can be quite potent and bipolar [205]: tumor microenvironments can, in some instances, function as a classic tumor promoter or co-carcinogen and potentiate the effects of a malignant epithelial phenotype, while in other instances, they can normalize the phenotype of epithelial cancer cells even though the genotype remains altered [212].

The CSC microenvironment thus shares similarities with normal stem cell niches. Epigenetic "cross-talk" between epithelial cells undergoing transformation and their surrounding stroma probably represents aberrations of normal "cross-talk" between these two populations [202]. In this cross-talk, a two-way clonal selection process may be occurring: aberrant stroma promotes the growth and development of transformed epithelial cells, while simultaneously, transformed epithelial cells promotes the development of aberrant stroma, as the two populations "walk down the transformational road" together [205]. For example, when the SV40T-immortalized-but-not-neoplastic human prostate epithelial cell line BPH-1 was grown in athymic mice in the presence of normal prostate fibroblasts, no tumor growth occurred. Similar results were obtained when normal human prostate epithelial cells were grown in the presence of human prostate carcinoma-associated fibroblasts (CAFs). However, when the BPH-1 epithelial cells were grown in athymic mice in the presence of human prostate CAFs, large and poorly-differentiated tumors developed [213]. Genetic alterations within stromal cells also play an important role in adjacent epithelial transformation. For example, a hereditary precancerous syndrome, juvenile polyposis, involves genetic mutations of a gene that is mainly expressed in the non-epithelial lamina propria of developing polyps [214]. In mice, heterozygous expression of the murine tumor suppressor neurofibromatosis gene ($NF\text{-}1^{+/-}$) in the stromal cells, surrounding Schwann cells in which both alleles of the NF-1 gene have been ablated ($NF\text{-}1^{-/-}$), significantly increases the frequency and size of subsequent neurofibroma formation compared with animals with a wild type stroma ($NF\text{-}1^{+/+}$) [215]. Finally, when a murine mammary epithelial cell line (COMMA-D cell line) harboring mutations in both alleles of p53 was transplanted into host mammary fat pads that had been exposed to ionizing radiation, the frequency and rapidity of tumor development as well as subsequent tumor size was greatly increased compared with un-irradiated hosts [216].

The recently-described "tissue organization field theory of cancer" [201,202] proposes a role for the complex CSC microenvironment that is very similar to the role of the niche in normal stem cell homeostasis. According to this theory, the normal "default" behavior of a cell is proliferation instead of the frequently assumed quiescence. Suppression of cell proliferation is achieved through cell adhesion-dependent tissue architecture (just as niches are required to maintain stem cells in a quiescent state). Therefore, the molecules and pathways that maintain normal tissue architecture, such as the adhesion molecules mentioned above, can be regarded functionally as forms of "tumor suppressors," as their loss or dysfunction removes inhibitory stimuli thus allowing cell proliferation and encouraging epigenetic tumor "progression" (just as stem cells leaving the niche transform into more rapidly proliferating progenitor cells). By the same token, restoration of tissue organization can repress the malignant phenotype of transformed cells, although in reality, this effect is probably attainable only when the cells are in the early stages of transformation and have not become too autonomous (just as early progenitors, but not fully differentiated cells, appear capable, in some instances, of reverting to full stem cell status when re-placed into the niche environment; Fig. 4). In this context, the tissue organization field theory of cancer does not contradict the somatic mutation theory, but complements it. The importance of epithelial stem cell acquisition of multiple genetic mutations during the transforming process may be seen as paramount but somewhat reductionistic; the tissue organization field theory expands the picture by describing how the environment in which mutational acquisition takes place, especially in the earliest stages of carcinogenesis, can play a critical role in enhancing or repressing the process and in determining the extent of phenotypic changes that result.

Several lines of data support the "tissue organization field theory" of cancer by showing that transformation of the complex microenvironment of a tumor can promote

progression, while re-creation of a more normal microenvironment can inhibit it. A number of striking examples of a normal microenvironment repressing a malignant phenotype exist. Illmensee and Mintz, in 1976, injected mouse teratocarcinoma cells that had been passaged for 8 years as in vivo ascites tumors into developing blastocyts, and completely normal chimeric animals resulted [217]. More recently, nuclear transfer of medulloblastoma nuclei into normal mouse oocytes produced normal mouse embryos [218], and nuclear transplantation of a Ras-inducible melanoma nucleus into mouse oocytes produced fully developed chimeric animals [219], which nevertheless developed melanomas and rhabdomyosarcomas at a much higher-than-normal rate. Collectively, these data indicate that, in the proper microenvironment, some malignant stem cells can be directed to fully differentiate into normal tissues, while maintaining their genetic mutations and inherent ability to develop malignancy. Consistent with the tumor-suppressing role of a normal microenvironment, matrix metalloproteinases (MMPs) frequently enhance tumor progression. MMPs have long been known to degrade extracellular matrix (ECM) molecules and remodel the ECM in a prolific fashion; they have recently been shown, in addition, to cleave receptors involved in cell adhesion, generate angiogenic factors, activate growth and death domain factors, select apoptosis-resistant subpopulations of cells and alter the biologic activities of ECM components. MMPs are frequently overexpressed during the transformation of epithelial cells (for recent reviews, see [220–223]). In mice, transgenic expression of the MMP stromelysin-1 within mammary glands leads first to the appearance of a reactive stroma [224] and then to the spontaneous development of premalignant epithelial changes, and subsequently, malignancy [225]. Thus, in many instances, the extensive alterations produced by MMPs in the stromal microenvironment of epithelial CSCs promote tumor progression.

3.8. The recently discovered plasticity of stem cells, potentially even across derivative embryonic layers, is observed in cancer as well

It has recently become apparent that many postnatal stem cells, usually in settings of tissue damage, regeneration or abnormal environmental signaling, have a surprising flexibility in forming other differentiated somatic cell types, even across derivative embryonic layers [226] (for reviews, see [227–229]). Thus, hematopoietic stem cells (HSCs) appear to have the capacity to generate hepatic oval cells [230], cardiac muscle cells [231] as well as epithelial cells of the liver, lung, gastrointestinal tract and skin [229,232,233]; mesenchymal bone marrow stem cells can generate neural cells [234], skeletal muscle cells [235] as well as adipocytes, chondrocytes, osteoblasts, tenocytes and smooth and cardiac muscle cells [228]; neural stem cells can generate a full lineage of hematopoietic cells [236], skeletal muscle cells [237], and when injected into blastocysts they form chimeric embryos that give rise to all three germ layers [238]; skeletal muscle stem cells can generate hematopoietic cells [239]; and finally, corneal epithelium can, under the influence of embryonic skin dermis, generate hair [240]. Additional examples of adult somatic stem cell plasticity will no doubt be generated, although its physiologic significance in the normal homeostatic setting of an organism is unclear. In addition, whether the mechanics of the process involve dedifferentiation, transdifferentiation or cell–cell fusion in individual settings has not yet been resolved [241–244].

Another form of epithelial plasticity, that has been described for some time, is termed *epithelial–mesenchymal transition* (EMT) (for reviews, see [245–249]). In most instances, EMT does not appear to involve true transdifferentiation, but rather, the temporary acquisition by epithelial cells of a motile, mesenchymal-like phenotype [250]. EMT occurs in embryogenesis, during gastrulation and the formation of various organs, and episodically again in adult life, when tissue damage necessitates epithelial cell movement during wound healing. In this process, cells within the organized "sheet-like" structure of normal epithelium lose their cell–cell contacts, along with their polarity, and undergo a dramatic remodeling of the cytoskeleton, as epithelial markers (E-cadherin, α- and γ-catenin) are repressed and mesenchymal markers (fibronectin, vimentin, smooth muscle actin, N-cadherin) are expressed [245]. Once requirements for motility are completed, the cells reacquire their non-motile, normal phenotype in the new location [246]. Many external signals appear able to initiate EMT, such as TGF-β in human keratinocytes [251], but loss of E-cadherin, a central building block of the adherens junctions that form lateral connections between epithelial cells, appears to be a key, and probably necessary, step in this process [245,248].

True metaplasia of CSCs has not yet been formally shown to underlie the development of any specific cancers (e.g., a mesenchymal neoplasm arising from a transformed epithelial progenitor cell or vice-versa). However, some data relating to CSC plasticity exist. Recently, gastric cancer was proposed to originate from bone marrow-derived mesenchymal stem cells [252], but the process involved two steps: first, migration of normal bone marrow-derived mesenchymal stem cells into regions of gastric ulceration undergoing stem cell loss due to chronic *Helicobacter* infection with subsequent formation of new gastric epithelium, and second, transformation of these new gastric epithelial cells into carcinoma. A similar two step process appears to have occurred in basal and squamous cell carcinomas arising in organ transplant patients that contain varying amounts of donor-related cells [253]. EMT is frequently observed during later stages of tumor progression in many epithelial cancers (for reviews, see [245,246,249,254,255]). In this setting, the reversibility of the process is not known. In fact, it appears that some of the carcinoma-associated fibroblasts present in the stroma surrounding epithelial cancers as well

as other settings of fibrosis, may, in fact, be of epithelial origin [250,256]. In addition, loss of E-cadherin expression and/or function correlates with tumor grade and worsened patient survival [254], and invasive potential [249] in a variety of epithelial cancers, including those arising in breast, prostate, colorectal and bladder tissues; concurrent activation of the ras and transforming growth factor-β pathways appears to synergistically evoke EMT in many cancers [246]; and finally, carcinosarcomas are rare human tumors having both epithelial and mesenchymal features [254] and stromelysin-1 expression causes the formation of infiltrative mesenchyme-like tumors in normal mammary epithelial cells, and the development of carcinosarcomas in transgenic mice [225].

To highlight the potential significance of stem cell plasticity in neoplastic transformation, we review the process within a specific epithelia, the epidermis. Normal skin stem cells show a capacity to form other differentiated cell types in response to microenvironmental signaling. In a porcine model of wounding, in the presence of remnant hair follicles, a completely normal stratified epidermis is reformed, but in the absence of follicular stem cells, a thin, metatypical epidermis can be constructed, apparently, from eccrine gland stem cells [257]. When isolated epidermal stem (but not TA or differentiated) cells from the skin of 3-day-old transgenic mice are injected into 3.5-day-old blastocysts, the stem cells are incorporated into ectodermal, mesodermal and neural crest-derived tissues and are still present in 60-day-old adult mice [258]. Dermal stem cells also show significant plasticity [198]. Cultured dermal papilla and dermal sheath cell lines can be induced to differentiate into adipocyte and osteocyte lineages [259]. When mouse dermal stem cells are isolated in a manner similar to the creation of neurospheres and mammospheres, they can subsequently be stimulated in culture to differentiate into neurons, glia, smooth muscle cells and adipocytes [260]. Strikingly, when cultured dermal papilla or dermal sheath cells are injected into lethally irradiated recipient mice, multi-lineage reconstitution of the entire hematopoietic system occurs [261]. Finally, we speculate that the rare setting of extramedullary cutaneous hematopoiesis [262] could reflect either homing of hematopoietic stem cells to skin, via the expression of the chemokine SDF-1 [263], or formation of an erythrocyte lineage from local dermal stem cells through as yet-unidentified signals. Little is known about the role that cancer stem cell (CSC) plasticity may play in the formation of various epidermal cancers, but several observations, and also speculations, may be made. Experimentally created mouse squamous cell carcinomas (SCCs) have been shown to undergo EMT and transform to an aggressive spindle cell phenotype. This is accompanied by a loss of E-cadherin expression [264] and a switch from stromal cell to tumor cell expression of stromelysin-1 [265]. E-cadherin expression also appears to be decreased in the infiltrating subtype of basal cell carcinoma, which has a more “mesenchymal” cell phenotype, but not in the superficial and nodular subtypes [266]. Atypical fibroxanthoma is an uncommon cutaneous malignancy with an unknown cellular origin [267]. It has been considered to be a variant of cutaneous SCC, an epidermal malignancy, as well as a superficial form of malignant fibrous histiocytoma, which is a sarcoma of mesodermal origin. Perhaps the tumor represents a SCC that has undergone some degree of EMT. Finally, it is well documented that cutaneous SCCs arising from sites of chronic, non-healing wounds are frequently anaplastic and show a high metastatic rate [54]. Perhaps, the chronic stimulus to undergo EMT in wound healing and the maintenance of a migratory phenotype selects for a pool of altered mesenchyme-like epidermal stem cells, so that when one undergoes further malignant conversion, it has already acquired the spindle cell features necessary for invasion and metastasis [268]. In addition, maintenance of the stem cells in a chronic proliferative state may ameliorate certain telomerase functions that have been theorized to protect undifferentiated stem cells from malignant transformation [269], and chronic activation may enlarge the pool of stem cells available for oncogenic change (Fig. 3) [27].

4. Implications for therapy

As noted in the Introduction, many current cancer therapies target the rapidly cycling transit amplifying/committed progenitor segments of cancer cell populations. This can lead to impressive, but often temporary, clinical remissions, as these rapidly-cycling cancer cells as well as their offspring committed to terminal differentiation – which together constitute the majority of the population – are eradicated. However, if the slow-cycling CSCs survive the therapy, they will eventually re-constitute the malignancy. Thus, to be effective, cancer therapy must target the CSCs, which are the ultimate reservoir of the cancer population. Several characteristics of CSCs may make them difficult to eradicate. First, they may be slow-cycling, and thus can survive many traditional chemotherapeutic regimens that target actively cycling cells. Second, many stem cells (and presumably CSCs) contain multi-drug resistance proteins [270] that protect them from chemotherapeutic and certain other environmental insults. Third, there are conflicting data regarding the sensitivity of stem cells (and presumably CSCs) to radiation therapy. Therefore, many new therapeutic avenues that specifically target the CSC population take advantage of the notion, discussed earlier in this paper, that microenvironmental signaling has the capacity to normalize the malignant phenotype of cancer cells, even though the existing mutations in their gene sequences remain unchanged. Finally, because of our growing awareness that stem cells begin to accumulate cancer-related mutations prior to the development of frank phenotypic cancer, and many cancer patients in whom field cancerization is occurring are at risk for the development of

subsequent and multiple carcinomas, the use of CSC-based therapies in an adjuvant, preventative fashion (termed *chemoprevention* [271]) holds great promise in individuals at high risk for cancer development.

4.1. Multi-drug resistance proteins

As noted above, many stem cells (and presumably CSCs) contain multi-drug resistance proteins that confer a survival advantage [270]. One of the defining features of many stem cells is the ability to transport and extrude Hoechst dye, and this has been termed the "side-population" (SP) phenotype [272]. Interestingly, although the SP phenotype has been identified with hematopoietic, muscle, neural [272] and corneal epithelial [273,274] stem cells, this feature does not seem to be associated with putative epidermal stem cell populations that have been identified as the "label-retaining cells" [275,276]. The physiologic basis of the SP phenotype is the presence of multi-drug resistance proteins, such as the multidrug resistance-1 gene product, p-glycoprotein and the breast cancer resistance protein [277]. These ATP-binding cassette transporter superfamily members are expressed at the highest levels on stem cells, to the degree that they are considered stem cell markers, and thus have implications for cancer therapy. They confer an ability to remove many cytotoxic (chemotherapeutic) drugs from the cytosol, causing a reduction in drug accumulation and increased drug resistance [278], and they are present in many human cancers [279]. In addition to conferring multiple drug resistance on stem cells and presumably CSCs, they also enhance cell survival in settings of hypoxia [280] and may provide resistance to certain forms of apoptotic-related cell death as well [277,278,281,282]. Between the survival advantage created by multiple drug resistance and their already slow-cycling nature, CSCs may be quite resistant to many chemotherapeutic regimens (unlike their rapidly-cycling and committed offspring), and new approaches that target CSCs specifically must be devised. Increased understanding of specific phenotypic and physiologic differences between CSCs and their normal stem cell counterparts (Table 4) may allow the creation of therapies that specifically target the former [283–285].

4.2. Irradiation

As noted above, another CSC therapeutic issue is the degree to which stem cells and CSCs are sensitive to the effects of irradiation. Accumulated data suggest that stem cells are much more sensitive to irradiation than differentiated cells [102], although there is also evidence that this population may be less sensitive to fractionated radiation doses than their more-differentiated offspring [286], and that their recovery may be assisted by neighboring, radioresistant cells [287]. Thus, the functional radiosensitivity of CSCs, relative to other tumor cell populations, remains unclear, and it is important to further study this subject in order to explore the radiotherapeutic management of CSCs.

Table 4
Normal stem cell and cancer stem cell markers

Organ	Normal stem cell	Cancer stem cell
Brain	CD133, msi-1, Sox2, melk, PSP, bmi-1, nestin side population [62–64]	CD133, msi-1 Sox2, melk, PSP, bmi-1, nestin side population [62–64]
Breast		$CD44^{+}$, $CD24^{-/low}$,4 $Lineage^{-}$ [30]
Epidermal hair follicle	K15, CD34, S100A4/A6, Myoc, Gpr49, Col6a1, Ltb2, Idb2, Dab2, Bdnf, Tek2, β6-integrin, Pdlim3 $CD\ 43^{+}$, $Nestin^{+}$, $K15^{-}$, [378,379]	
Gut	msi-1, hes-1 [6,7]	
Hematopoietic	Lin^{-}, $Thy1^{-}$, $CD34^{+}$, $CD38^{-}$, $CD71^{-}$, $HLA\text{-}DR^{-}$ [1724]	Lin^{-}, $Thy1^{-}$, $CD34^{+}$, $CD38^{-}$, $CD71^{-}$, $HLA\text{-}DR^{-}$, [22] IFNRN-1, DAPK-1, Mcl-1, NF-κβ, heat-sensitive [283,285,306,307]
Prostate	CD133 [380]	

Bmi-1=a Polycomb Group gene required for HSC self-renewal and proliferation.
Col6a1=procollagen, type VI, alpha 1.
Dab2=disabled homolog 2.
DAPK-1=death-associated protein kinase-1.
Grp49=G protein-coupled receptor 49 FEX.
Hes-1=hairy and enhancer of split homologue-1.
Idb2=Id binding protein-2.
IFNRF-1=interferon regulatory factor-1.
Melk=maternal embryonic leucine zipper kinase.
Msi-1=musachi-1.
Myoc=trabecular meshwork induced glucocorticoid protein.
PSP=phosphoserine phosphatase.
side population=unlabelled after extrusion of Hoechst 3342 dye.
Sox2=an early transcription factor expressed in NSCs and the developing neural tube.

4.3. Differentiation, apoptosis and senescence

A number of therapies currently being developed focus their effects on CSCs, by promoting their terminal differentiation along more normal pathways, or in some instances, stimulating CSC apoptosis through microenvironmental cues [212,288]. As an extreme example, noted earlier, when multiple-passaged mouse teratocarcinoma cells were injected into developing blastocysts, these cells were incorporated into various normal tissues in normal animals, indicating that the differentiation pathway of at least some CSCs can be restored by proper microenvironmental signaling [217]. It should be noted that both terminal differentiation and apoptosis (including related forms of active cell death such as mitotic catastrophe and micronucleation) as well as cellular senescence all appear to involve overlapping pathways, and many of the cytodifferentiation therapies described below frequently involve the

recruitment of CSCs into more than one pathway, depending on the specific setting involved [289,290].

There are several examples of "normalization therapies" for cancers: (i) The differentiation-promoting agent all-trans retinoic acid (ATRA) is now considered a part of the standard treatment regimen for acute promyelocytic leukemia (APML) [291], which appears to arise from a committed myeloid progenitor. ATRA induces complete remission in a majority of APML patients, and the mechanism of action seems to be the induction of terminal differentiation as well as apoptosis of the malignant clones [291,292]. (ii) Other retinoids, in a number of studies, also appear to normalize differentiation at the stage of dysplasia, in oral leukoplakias [271]. (iii) Histone deacetylases (HDACs) regulate gene expression at the transcriptional level in cells by removing acetyl groups in nucleosomal histones. Deletions and inactivating mutations in HDACs are associated with human cancer formation [293]. HDAC inhibitors have been shown to induce senescence, apoptosis and/or terminal differentiation in many tumor cell lines [294]. A phase I clinical trial of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) in humans showed antitumor activity with a favorable side effect profile [295]. SAHA has been shown to be a potent inducer of apoptosis specifically in multiple myeloma cells [293,296]. (iv) Mutations in the PDGF-PDGF receptor system appear to underlie the development of dermatofibrosarcoma protuberans (DFSP), an uncommon sarcoma of dermal origin. Nearly all cases of DFSP involve a chromosomal rearrangement of chromosomes 17 and 22, resulting in a fusion of the collagen IαI gene with the gene for the PDGF β-chain. This leads to over-activation of the PDGF receptor for the β-chain in the mesenchymal cells, which appears to be a central oncogenic stimulus for development of this tumor. Imatinib, a potent and specific inhibitor of the PDGF receptor, has shown promise in both preclinical as well as clinical studies in the treatment of DFSP [297,298]. (v) Constitutive Ptch–Hh–Gli pathway over-expression appears to be requisite for basal cell carcinoma (BCC) development, and has been noted in some medulloblastomas as well. Inhibition of this pathway with the agent cyclopamine, which blocks the Hedgehog pathway at the level of Smoothen (Fig. 7), has been shown in preclinical models to inhibit proliferation, induce differentiation and cause significant cell death in medulloblastoma tissues [299]. Use of a similar Hedgehog pathway inhibitor in preclinical models of BCC caused complete apoptosis of tumor nests without detrimentally affecting surrounding normal keratinocytes [300]. However, it is possible that these Hh pathway inhibitors may affect the rapidly-cycling BCC transit-amplifying cells but not the slow-cycling CSCs. In one study involving transgenic mice [301], constitutive Hh pathway expression produced BCC-like tumors, while subsequent inhibition led to their almost complete involution. However, a small subpopulation of quiescent tumor cells persisted for months that were thought to represent the CSCs. Subsequent re-activation of the Gli transgene led to tumor re-formation from these cells, indicating that they had "escaped" the effects of prolonged pathway inhibition. (vi) Troglitazone, which is a ligand for the peroxisome proliferator-activated receptor-γ, has been used in the treatment of liposarcoma [302]. In early preclinical work, troglitazone was shown to induce lineage-appropriate differentiation in intermediate-to-high grade liposarcomas [302], although in a more recent phase II clinical trial, no clinical improvement was noted [303]. (vii) Finally, therapeutic efforts at the level of pre-cancerous fields is still in its infancy [271]. Some of the most promising recent work has involved the use of retinoids in head and neck precancer, while topical 5-fluorouracil has been used by dermatologists to treat areas of extensively sun-damaged skin for decades. Such therapy both eradicates pre-cancerous actinic keratoses and dramatically reduces their subsequent formation, often for a period of several years. Collectively, the therapeutic agents discussed above represent an approach to the treatment of cancer and precancerous fields that seeks to induce differentiation, cell death and/or senescence in CSCs and their offspring at the earliest developmental stages.

5. Still-unanswered questions about CSCs

5.1. How to identify CSCs?

In several organs that have been investigated, CSCs appear to have a phenotype very similar to that of normal stem cells. Thus, in acute myeloid leukemia (AML) CSCs capable of transferring the disease to an appropriate host express the normal hematopoietic stem cell (HSC) [$Thy1^-$, $CD34^+$, $CD38^-$] phenotype [22]; and in pediatric brain tumors, almost all proliferative activity is found in the small subset of malignant cells that express the neural stem cell marker CD133 [62]. In addition, like normal stem cells CSCs appear to be slow-cycling [304,305], and constitute only a small percentage of the total tumor cell population [30,58,62]. However, when CSCs and normal stem cells from a given organ are compared, differences do exist (Table 4). For instance, while CSCs of AML share many surface markers [$CD34^+$, $CD38^-$, $CD71^-$, HLA–DR^-] with normal HSCs, three surface markers [$CD90^-$, $CD117^-$ and $CD123^+$] seem to be unique to the CSC population [283]. In addition, CSCs from AML patients express the tumor suppressor genes interferon regulatory factor-1 and death-associated protein kinase-1 [306], the anti-apoptotic factor Mcl-1 [283] and a constitutively active survival factor NF-κβ [307], none of which are found in normal HSCs. CSCs from AML patients also appear to be much more sensitive to heat than normal HSCs [285]. Thus, the ability to identify CSCs within a given organ tends to parallel our ability to identify normal stem cells within that system. Therefore, it is important to

continually utilize newly discovered stem cell markers [308,309] to identify possible CSCs using immunohistochemical staining and cell sorting. The identification and isolation of CSCs will make possible further studies of the peculiar growth and differentiation properties of the CSCs. In addition, they will allow the answer of many important questions: Within tumors, are CSCs clustered or are they dispersed as single cells? If CSCs are clustered, do they interact with a specialized CSC niche? And how do the CSCs respond to various therapeutic treatments?

5.2. Why don't traditional chemotherapies eventually eradicate CSCs?

Although we state that traditional chemotherapies target rapidly cycling cells, and this would make them unlikely to have a significant effect on the slow-cycling CSC population, this is not necessarily the whole story. We know that following wounding, stem cells in normal epithelia do begin to cycle in response to the loss of the terminally differentiated cell population (Figs. 3 and 4) [13,106,310]. Thus, although an initial application of chemotherapy or radiation should theoretically kill only the rapidly-cycling TA cells, repeated applications should eventually deplete the stem cell population as well. One possible explanation for this discrepancy is that the signaling mechanisms which initiate stem cell cycling in normal populations in response to tissue loss, perhaps mediated by the surrounding niche, are not functional or present in the CSC microenvironment. Another is that the effects of the drug resistance ABC proteins are overriding; perhaps even when they are stimulated to proliferate, CSCs are well protected by the multi-drug resistant genes.

5.3. During the formation of patches and fields, how do early epithelial CSCs, which are slow-cycling and have an undifferentiated phenotype, migrate?

Migratory capability in normal adult epithelium, for instance following wounding, involves the acquisition of a temporary migratory phenotype. However, following wound healing (involving migrating normal epithelial cells), or the formation of patches and fields (involving migrating pre-malignant epithelial cells), an undifferentiated stem cell phenotype must at some point be re-established. How this occurs is unknown. One possibility is that the creation of a niche microenvironment restores upon some of the motile cells a "stem-cell status," (Fig. 4) but the mechanisms involved have not yet been examined.

5.4. How do differentiation-promoting agents remove CSCs from a tumor population?

Replication of normal stem cells maintains two important populations: the stem cell population itself and its offspring, the transient amplifying and terminally differentiated cells, that make up the bulk of every organ (Figs. 1–3). The same appears to be true of CSCs. The regulatory mechanisms involved in controlling these two populations in either the normal or the malignant setting are not well understood (Fig. 3). However, no diseases or circumstances are known to exist that result in a total progression of normal stem cells into committed cells with an accompanying loss of the underlying stem cell population; intuitively, strong mechanisms must exist to prevent such an event. Nevertheless, if a therapeutic agent induces the true loss of all CSCs through terminal differentiation, this is exactly what would need to occur. Another possibility is that these agents alter the microenvironment of the CSCs so that they "behave" more normally, even though they retain their genetic mutations and hence their capacity for malignant behavior (e.g., [217–219]). Finally, as was discussed earlier, most of these agents appear to drive malignant clones down multiple pathways simultaneously: towards terminal differentiation, apoptosis and related pathways of active cell death, and cellular senescence [289,290]. The relationships that exist among these different pathways, and the mechanisms by which one is turned on versus another, are just beginning to be understood, and will hopefully contribute to improved therapeutic options for eradicating CSCs.

5.5. How are different types of cancers produced in one organ—by malignant conversion of different types of stem cells, or by the acquisition of a different "package" of genetic mutations in the same type of stem cell?

A number of in vitro studies suggest that the specific cell type undergoing malignant transformation affects both the likelihood that cancer will ultimately develop, as well as the type that occurs [29]. We will discuss the principles involved in this process using the skin as an example. *First*, significant evidence indicates that stem cell populations exist in both follicular and interfollicular regions of skin, although the follicular bulge stem cells are regarded by some as the 'ultimate' skin epithelial stem cells (reviewed in [3,13,310–313]). While it appears that the follicular bulge reservoir may function to repopulate interfollicular skin following wounding [310], the precise relationship between these two cell populations during periods of normal homeostasis is unclear [311,312,314,315]. In a large body of work developed over decades, researchers have attempted to determine whether progenitor cells from follicles, interfollicular epidermis or both are a source of skin cancer formation (reviewed in [316]). Several avenues of approach have been used, including (i) histologic and/or histochemical examination of the earliest precancerous and cancerous lesions [317–326], (ii) superficial irradiation of skin to a depth that includes or does not include the hair follicle bulge [327–329], (iii) two-stage carcinogenesis studies following abrasion that removes interfollicular epidermis but leaves behind hair follicles [330–332], (iv) carcinogenesis studies in hair-bearing versus hairless settings [333–335], (v) skin grafting studies in which

isolated follicular or interfollicular components are exposed to tumor initiators and promoters [336,337], (vi) two-stage carcinogenesis protocols performed during specific periods of the hair cycle [338–343], (vii) assessment of the cellular location of carcinogen persistence [316,344–346], and most recently, (viii) experiments involving transgenic mice, in which mutated genes are expressed in specific populations of cells (e.g., follicular, basal or suprabasal) depending on the promoter used [301,347–351]. In general, this body of work suggests that follicular progenitor cells are more likely to undergo malignant change than interfollicular progenitor cells but both may be targets, and basal cell carcinoma may be more closely related to hair follicle structures while squamous cell carcinoma may be more closely related to interfollicular epidermis. *Second*, when placed under control of either K5 or K14 (basal layer) promoters, overexpression of different signaling pathway genes produces different skin cancers in transgenic mice: stabilized β-catenin produces follicular tumors [152,154,352]; Lef-1 lacking a β-catenin binding site produces sebaceous tumors [160,353]; Gli1 and Gli2 produce basal cell carcinomas [173,183,184]; and mutant Harvey Ras produces papillomas and squamous cell carcinomas [348]. Similarly, in carcinogenesis studies in rats, topical treatment with anthramine or 3-methylcholanthrene produce mostly basal cell carcinomas, whereas topical treatment with 7,12-dimethylbenz(a)anthracene produces mostly squamous cell carcinomas [354]. *Third*, in two-stage carcinogenesis protocols involving mouse skin, subpopulations of papillomas that have a high or a low risk of malignant conversion to carcinomas can be produced [355]. In comparison to low risk papillomas, the high risk groups show suprabasal expression of $\alpha_6\beta_4$ integrin [356,357], significant suprabasal layer proliferation [356,358], loss of keratin 1 expression [356,357], loss of transforming growth factor-β expression [358,359] and decreased suprabasal expression of E-cadherin [360]. Unfortunately, none of these current data explain specifically how different types of cancers (BCC, SCC) can evolve from a single epithelia (the skin). If different epidermal stem cells types are equally susceptible to the effects of a specific transgene or carcinogen, then a given cancer could arise from either stem cell type; on the other hand, a different responsiveness of specific stem cell populations to the effects of a given transgene or carcinogen could also explain all of the above-noted results. The development of specific markers for the different types of epithelial stem and progenitor cells is required before a true assessment of the cell-of-origin of a given cancer can be made.

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