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Stem Cells Review Series

J. Cell. Mol. Med. Vol 10, No 2, 2006 pp. 292-308

Repair and regeneration: opportunities for carcinogenesis from tissue stem cells

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Received: February 10, 2006; Accepted: April 3, 2006

Abstract

This review will discuss the mechanisms of repair and regeneration in various tissue types and how dysregulation of these mechanisms may lead to cancer. Normal tissue homeostasis involves a careful balance between cell loss and cell renewal. Stem and progenitor cells perform these biologic processes as the functional units of regeneration during both tissue homeostasis and repair. The concept of tissue stem cells capable of giving rise to all differentiated cells within a given tissue led to the concept of a cellular hierarchy in tissues and in tumors. Thus, only a few cells may be necessary and sufficient for tissue repair or tumor regeneration. This is known as the hierarchical model of tumorigenesis. This report will compare this model with the stochastic model of tumorigenesis. Under normal circumstances, the processes of tissue regeneration or homeostasis are tightly regulated by several morphogen pathways to prevent excessive or inappropriate cell growth. This review presents the recent evidence that dysregulation of these processes may provide opportunities for carcinogenesis for the long-lived, highly proliferative tissue stem cell population. New findings of cancer initiating tissue stem cells identified in several solid and circulating cancers including breast, brain and hematopoietic tumors will also be reviewed. Finally, this report reviews the cellular biology of cancer and its relevance to the development of more effective cancer treatment protocols.

Keywords: cancer stem cell • tissue stem cell • cellular hierarchy • regeneration • cancer

Normal tissue repair and homeostasis

Normal tissue homeostasis involves a careful balance between cell loss and cell renewal. In order to maintain this balance, tissues must possess cells capable of self-renewal as well as differentiation. Stem and progenitor cells perform these biologic processes as the functional units of regeneration

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Reprinted from:
Journal of Cellular and Molecular Medicine
Published by:
the CMM Foundation

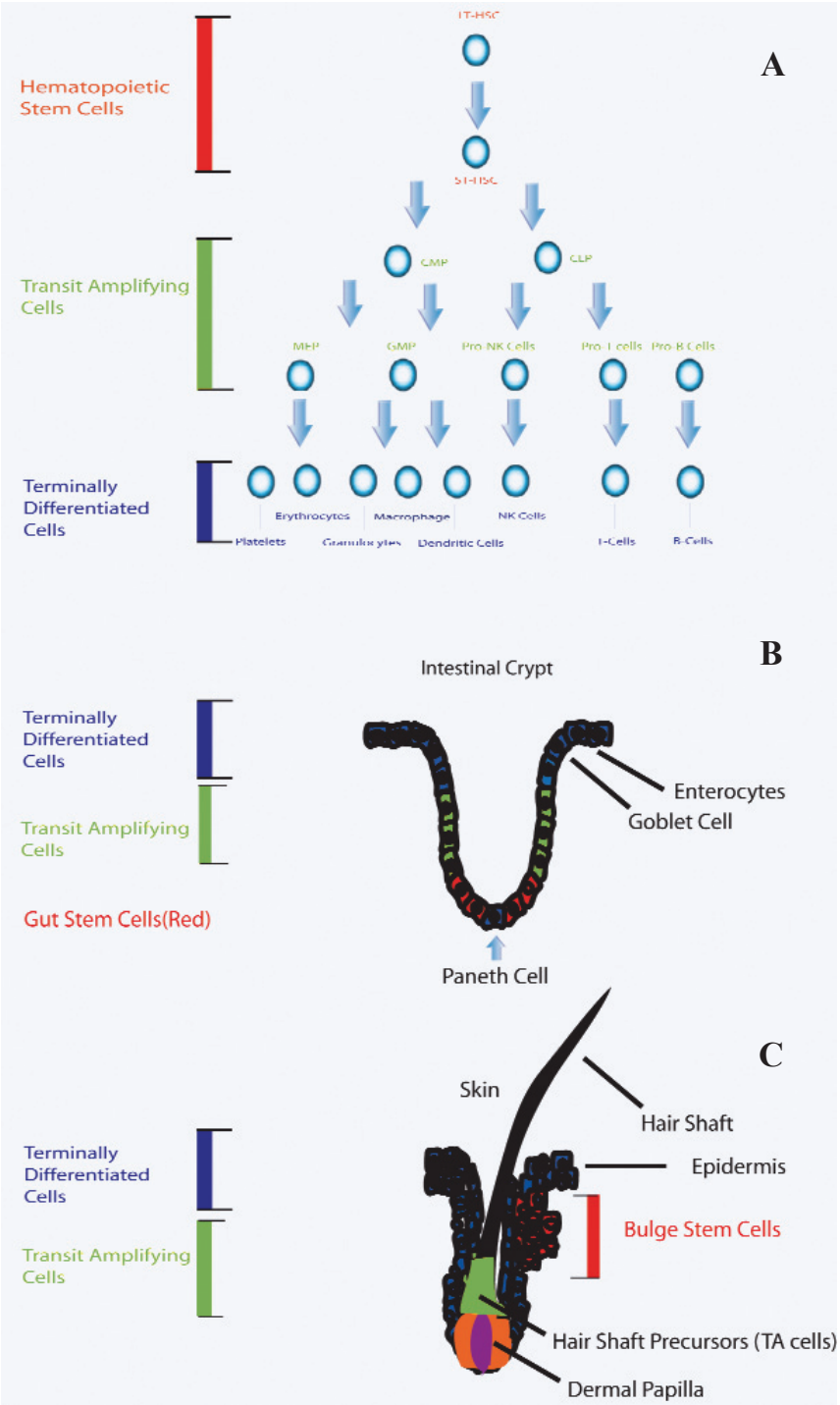

Fig. 1 Cellular hierarchy within the hematopoietic (**A**), intestinal (**B**) and skin (**C**) tissue compartments. (**A**) In the hematopoietic system, the long-term and short-term repopulating stem cells give rise to early progenitor cells. These cells are highly proliferative and are known as transit amplifying cells. They are able to divide a limited number of times before terminally differentiating to effector cells (erythrocytes, macrophage, platelets etc). (**B**) In the gut, stem cells are located near the base of the Crypts of Leiberkühn. Gut stem cells differentiate as they migrate upwards to the surface epithelium. Before terminally differentiating into absorptive enterocytes or secretory goblet cells, progenitor cells give rise to transit amplifying cells, the immediate precursors to the effector cells. (**C**) Finally, skin stem cells are located within the bulge region of the skin follicle. Signals from the dermal papilla lead to proliferation of bulge stem cells. As in the hematopoietic and intestinal compartments, the progenitor stem cells give rise to transit amplifying cells located within the hair bulb on their way to differentiating to skin effector cells.

during both tissue homeostasis and repair. Tissue specific stem cells were first described in the hematopoietic system by Gilbert and Lajtha in 1964. They introduced the concept of a multipotent stem cell capable of both self-renewal and of giving rise to more differentiated progeny [1]. These long-term repopulating stem cells are by definition capable of the complete reconstitution of all the differentiated cell types in the hematopoietic system (Fig. 1A). From this initial discovery has grown the concept of a "cellular hierarchy" within tissues. At the apex of this hierarchy is a resident tissue stem cell. This cell is undifferentiated yet capable of an asymmetric mitosis producing a replica cell and a more

committed progeny cell. To date the hematopoietic system remains the best characterized post-natal stem cell compartment and thus serves as a model system to which other stem cell systems are often compared. As such, it is instructive to have a working knowledge of the cellular hierarchy that exists within the bone marrow (Fig. 1A). The most primitive cell types at the pinnacle of the hematopoietic system are the long-term and short-term repopulating hematopoietic stem cells (HSC) that give rise to more differentiated progeny; specifically, the common myeloid (CMP) and common lymphoid progenitor (CLP) cells. These cells, known as transit amplifying (TA cells), are highly proliferative and can in turn give rise to fully differentiated blood cells. In contradistinction to the HSC, TA cells are not capable of self-renewal. The HSC is therefore necessary and sufficient for long term reconstitution of ablated bone marrow.

A similar concept of tissue stem cell hierarchy exists in the gut where enteric crypt progenitor cells are also able to give rise to the multitude of differentiated gut epithelial cell types (Fig. 1B). In this system, stem cells are found near or at the base of invaginations within the epithelial lining of the gut, termed Crypts of Lieberkühn [2]. Similar to the HSC, the gut stem cell is capable of self-renewal and the derivation of transit-amplifying (TA) progenitor cells. In contrast to the hematopoietic system, the subsequent divergence of lineage committed TA cells in the gut has not been defined. As enteric progenitor cells migrate upward through the epithelium toward projections known as villi in the small intestine or toward the surface epithelium of the colon, they differentiate into functional absorptive and secretory intestinal cells [3]. These effector cells are eventually shed into the gut lumen and are replaced by new cells as the cycle of migration, functional differentiation, and loss is initiated repeatedly by the niche of renewing enteric progenitors. While cells within a single crypt may be derived from a single parent stem cell, multiple crypts contribute to the surface epithelium of a single villus resulting in a multi-clonal population of terminally differentiated effector cells lining the enteric surface epithelium. Therefore, as in the hematopoietic system, the gut stem cell is necessary and sufficient for the regeneration of the intestine following injury and its maintenance during periods of homeostasis.

In the skin, resident stem cells are located within the bulge region of the hair follicle and through asymmetrical division are able to self-renew and give rise to more differentiated skin progeny cells (Fig. 1C) [4]. Complete epithelial turnover in the skin occurs over 7 days in mice and 2 months in humans [5, 6]. As in the intestinal and hematopoietic systems, the bulge stem cells give rise to the secondary germ that in turn give rise to rapidly proliferating transit-amplifying cells. These committed progenitors in turn give rise to the terminally differentiated effector cells of the skin and skin appendages, most notably, the hair germ [7]. It has, however, recently been shown that these same bulge derived progenitors cells contribute to epidermal restoration during the wound-healing response, yet do not participate in epidermal homeostasis [8]. Under physiologic conditions of epidermal renewal, cells arranged into epidermal proliferative units are charged with maintaining the epidermis, but not the hair follicles. In response to signals from the dermal papilla, the long-lived epithelial stem cells in the bulge respond by deriving short-lived facultative “transit amplifying” cells responsible for maintaining the hair growth cycle [9]. Interestingly, fully differentiated keratinocytes contained in the secondary germ are capable of dedifferentiation to stem cells when bulge cells are depleted as a result of severe depilation-induced injury [10].

In contradistinction to the rapidly renewing tissue compartments discussed above, parenchymal regeneration within the liver proceeds by a different mechanism. During periods of physiologic homeostasis, the liver parenchyma remains relatively mitotically quiescent despite a high metabolic rate. During or following periods of injury, differentiated, quiescent hepatocytes are able to leave this state of cell cycle arrest and undergo DNA synthesis and cellular hypertrophy to replace lost cellular mass(Fig. 2A). As much as two thirds of the mouse or human liver can be removed with complete restoration of parenchyma within days or weeks respectively [11, 12]. This is done mostly by the resident hepatocytes with a small contribution from Kupffer, biliary and endothelial cells. The liver is unique among epithelial organs in its ability to restore a significant loss of parenchyma.

For hepatocytes, the predominant regenerative mechanism appears to be cellular hypertrophy that occurs as a result of cellular endoreduplication with or

without cytokinesis. The net effect of this process is an overall increase in the cell soma and DNA content resulting in large hepatocytes with polyploidy that may be multinucleate. In the case of more sustained chronic liver injury, when hepatocytes are unable to enter the cell cycle, a second compartment of cells actively proliferates and is capable of restoring damaged liver mass (Fig. 2B). These facultative progenitor cells, commonly referred to as oval cells, are located within the terminal bile ducts and are able to give rise to both hepatocytes and cholangiocytes [13]. These cells have been considered to be the transit-amplifying cells of the liver in cases of extreme tissue loss or impaired hepatocyte function. Oval cells have been identified in numerous rodent models of liver regeneration and repair as well as in human specimens of chronic pathologic liver disease states [14–17]. Despite this, little is known about the molecular mechanisms governing oval cell biology. However, several well-described morphogen signaling pathways including both the Wnt/β-catenin and Jagged/Notch morphogen pathways have begun to be characterized as critical factors in determining hepatic form and function throughout liver embryogenesis [18–20]. It is interesting to speculate that as a facultative transit-amplifying progenitor cell population, oval cells recapitulate the embryonal processes of hepatoblast proliferation and differentiation.

Tissue regeneration and cancer: the cancer stem cell paradigm

There is accumulating evidence that loss of control over normal tissue repair or renewal mechanisms

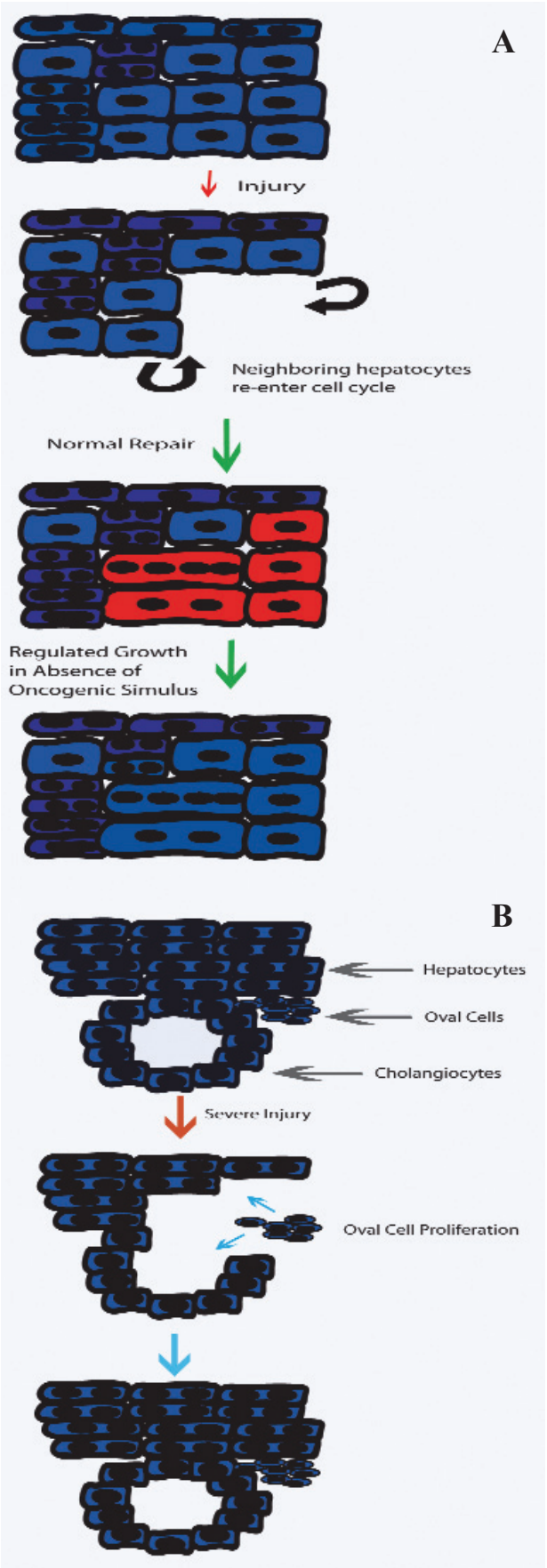

Fig. 2 Repair of acute liver injury by hepatocytes (**A**) and facultative transit amplifying progenitor cells (**B**). (**A**) When normal hepatocytes are lost through exposure to mechanical or chemical injury, neighboring hepatocytes re-enter the cell cycle in order to restore cell mass. These cells undergo a process of endoreduplication whereby nuclear DNA content is doubled and cell size increased with or without cytokinesis. Thus, liver mass may be restored by cellular hypertrophy. (**B**) In the case of severe or chronic liver injury, hepatocyte proliferation is impaired and liver mass is restored by a separate compartment of facultative transit amplifying progenitor (oval) cells. These cells are bipotent progenitors that give rise to cholangiocytes and hepatocytes.

may lead to malignant transformation. Cancer has therefore been described as a "wound that does not heal" or perhaps more appropriately, "the wound is a tumor that heals itself" [21, 22]. The association between cancer and persistent inflammatory or regenerative states may be a reflection of this possibility [23–25]. Moreover, highly conserved morphogen signaling pathways such as Wnt/β-catenin, Hedgehog, Jagged/Notch, FGFs, and the BMP/TGF-β superfamilies are all recapitulated in the process of postnatal tissue restoration, and when dysregulated, perhaps initiate carcinogenesis. Interestingly, consistent with this relationship between tissue repair and cancer is the observation that long-lived organisms and/or tissues with high rates of renewal such as the lung, gut, and blood also have the highest cancer incidence [26]. In contradistinction, juvenile cancers occur in rapidly expanding neural tissue (medulloblastoma, neuroblastoma) and parenchymal organs (hepatoblastoma, nephroblastoma) that are undergoing tissue expansion as an extension of the embryonal processes initiated during organogenesis.

Further evidence for an association between cancer and deregulation of regenerative mechanisms stems from the established increased cancer risk associated with aging. It is becoming increasingly recognized that there exists an inverse relationship between the regenerative capacity of stem cell compartments and the aging or quiescence of a given tissue [27]. Moreover, this relationship can be extended to the susceptibility of aging tissues to malignancy. Several investigators have begun to describe a shift in the balance between stem cell proliferation and quiescence gene pathways associated with aging. For example, adult tissue stem cells may be more sensitive to senescent pathways which must be activated as a cell compartment ages and accumulates genetic or epigenetic abnormalities in order to prevent progression to oncogenesis [28]. By the same argument, embryonal tissue, or more specifically, embryonal tumors of the young that arise from highly proliferative progenitor cells may be somewhat more sensitive to oncogenic activation and relatively resistant to dominant tumor suppressor/cell senescence pathways like p53, Rb and PTEN [29]. Others have argued that the late life phenotype that is intended to prevent carcinogenesis may be directly related to a diminished capacity in physiologic regenerative processes. Thus, as the organism's inherent ability to appropriately regenerate weakens, its propensity for cancer strengthens. It is therefore not surprising that the increased risk of epithelial cancers tends to accompany the aging process while non-epithelial cancers (those associated with genetic predisposition or early high-risk exposures) tend to predominate in juveniles [26].

Understanding how tissue regeneration may lead to cancer may require a shift in perspective toward viewing the regenerative processes that govern tissue restoration as providing opportunities for carcinogenesis (Fig. 3). Until recently, researchers had been mostly focused on the molecular biology of tumorigenesis without much regard for the implications of the founding cell population. However, the prospective identification of cancer initiating cells within the circulating tumors of the hematopoietic system and within solid organs such as the brain and breast has broadened our focus to include an understanding of the cells involved in initiating and propagating cancer [30–33]. Out of this changing paradigm was derived the concept of tissue stem cells serving as cancer stem cells.

Though tumors are populated by a heterogeneous group of cells, only a small subset of cells appears to have the ability to initiate and maintain this cancerous growth (Fig. 4) [30–32]. Previously, tumors were thought possibly to derive from any if not all of the cells contained therein. Based on this "stochastic model" of tumor development, any cell within a heterogeneous tumor population could both initiate new tumors and propagate them as metastases. However, serial tumor transplant experiments have shown that only a few cells are able to fully recapitulate tumors (Fig. 5) [31, 32]. These findings have led to a new paradigm of tumor initiation referred to as the "hierarchical model" of oncogenesis. In this model, tumors though heterogeneous, derive from a small subset of cancer initiating cells. In the same way that a tissue stem cell remains at the top of a tissue hierarchy, cancer initiating cells or cancer stem cells, are the subset of cells that give rise to the heterogeneous population of cells within a tumor. Thus tumors, like tissues may also be governed by a cellular hierarchy that is conveyed upon a small long-lived subset of cells by the gain of function ability to self-renew. This population is often found to represent less than 5% of the tumor mass and is necessary and sufficient for regeneration of the tumor.

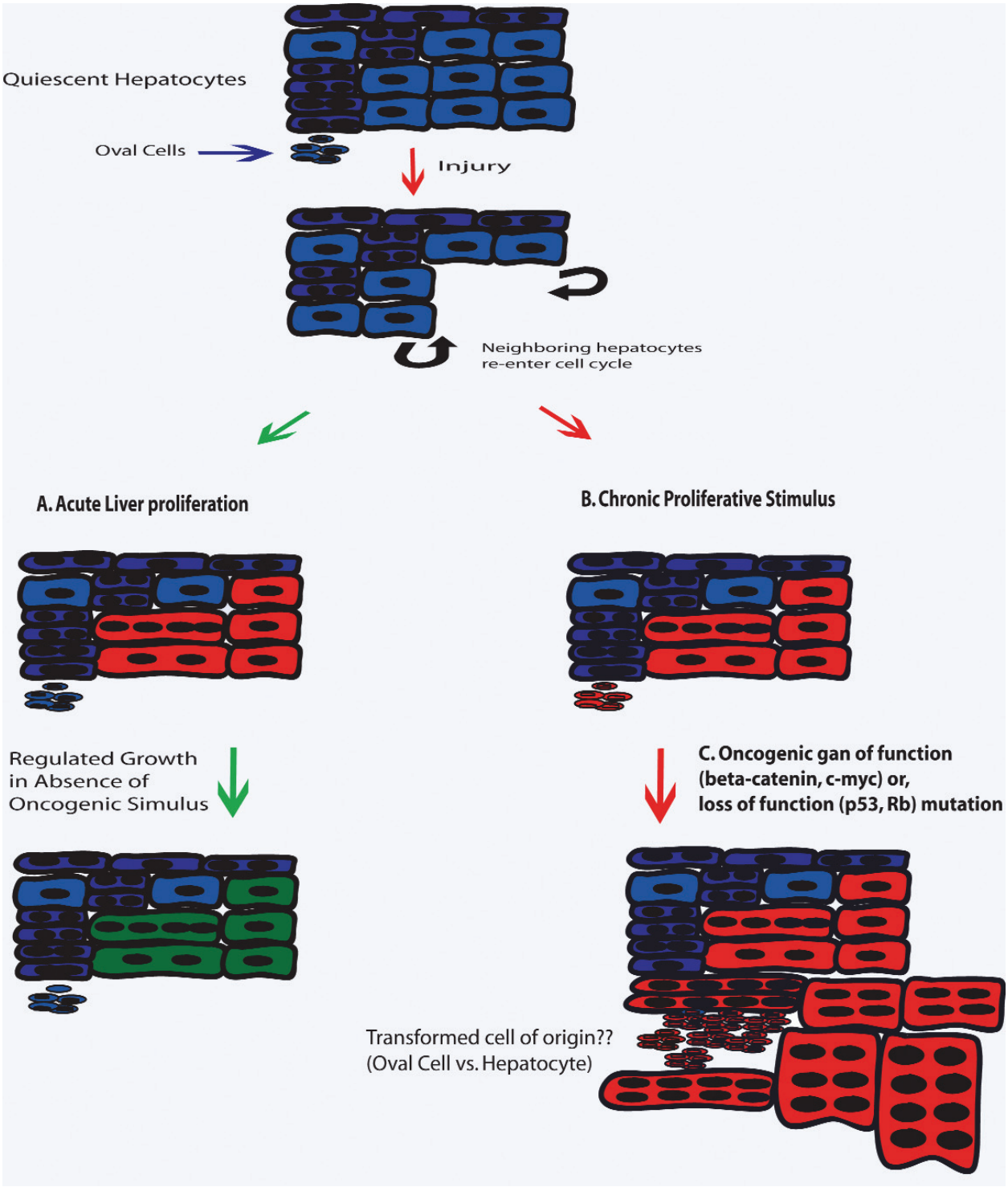

Fig. 3 Dysregulated tissue repair mechanisms in the development of cancer. In normal liver repair (**A**), neighboring hepatocytes replace lost organ mass. (**B**) In the presence of chronic proliferative stimuli, this repair process may be dysregulated and can lead to excessive proliferation and malignant transformation. This transformation may take the form of gain-of-function (β-catenin or c-myc) or loss-of-function (p53, Rb) mutations. The transformed cell may be from the hepatocyte or oval cell compartment.

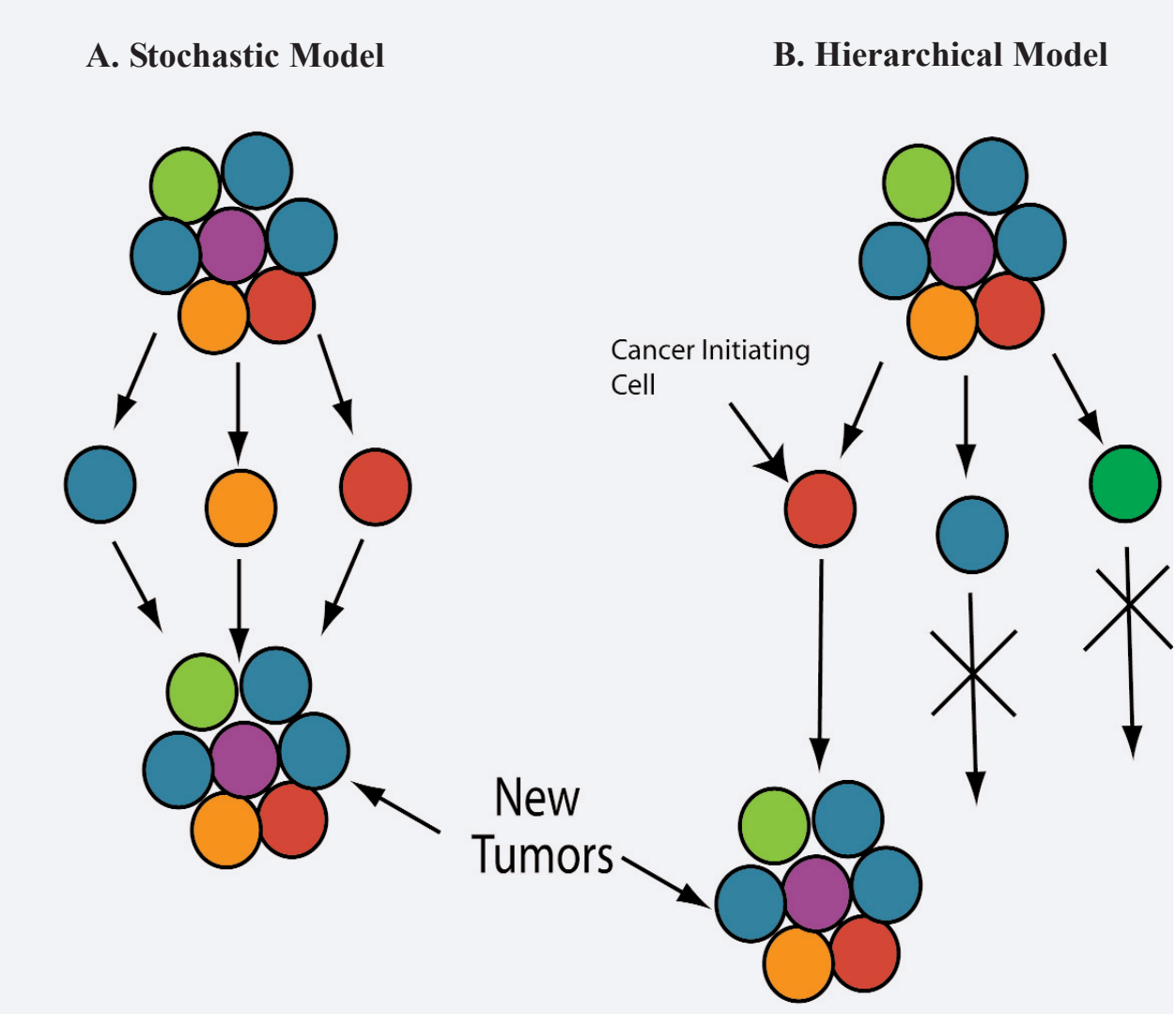

Fig. 4 Stochastic versus hierarchical model of carcinogenesis. In the stochastic model of carcinogenesis, cells from a heterogeneous tumor are each able to randomly give rise to new cancers. In the hierarchical model of carcinogenesis, only a small group of cells, known as the cancer stem cell (CSC) are able to give rise to new tumors. These cells represent a small fraction of the total tumor mass (<5%).

The proof of this concept of cancer initiating cells was first established in acute myelogenous leukemia (AML) [30]. Subsequent to this discovery, Bonnet was able to show that the SCID-leukemia-initiating cell or SL-IC was found only in the $CD34^{++}CD38^{-}$ fraction of bone marrow. This population is highly enriched for HSCs [34]. The $CD34^{++}CD38^{+}$ fraction, which is highly enriched for more differentiated progenitor cells or transit amplifying cells, was not capable of giving rise to AML. These important experiments provided evidence that in the case of AML, a primitive stem cell rather than a more committed cell was the target of leukemic transformation.

This result notwithstanding, Cozzio and Huntly each separately examined the ability of more committed progenitor cells to give rise to AML [35,36]. Given the strong correlation between the expression of certain fusion proteins derived from chromosomal translocations and the incidence of these leukemias, these groups examined the ability of two different fusion proteins to transform progenitor cells at various differentiation stages. Both groups found that transduction of both committed and

uncommitted progenitor cells with *MLL-ENL* and *MOZ-TIF2* fusion genes led to transformation of hematopoietic stem cells (HSCs), Common Myeloid Progenitors (CMPs) and Granulocyte-Monocyte Progenitors (GMPs) *in vitro*. Moreover, *in vivo* transplantation of these transformed cells led to AML in mice with the same latency for all three groups of cells. Given the long life of these transduced CMP and GMP lineage committed cells *in vitro*, it would seem that they had gained the ability to self-renew like the HSC. When these cells were transduced with another known leukemic fusion oncogene, *BCR-ABL*, only the HSC was transformed and able to give rise to AML *in vivo*. *BCR-ABL* is an oncogene that is known to provide a proliferative and survival advantage to progenitor cells. It is not capable of conferring the ability to self-renew onto the lineage committed progenitor cells. However, MLL-ENL and/or MOZ-TIF2 fusion proteins impair differentiation and are able to confer self-renewing properties onto these lineage-committed progenitor cells. Taken collectively, these results confirm that transformation of a long lived stem cell, or of a progenitor cell that has gained the ability for self-renewal, is required for the process of cancer initiation. Furthermore, these discoveries have led to speculation about the existence of tissue stem cells in other tissues which might also, given the appropriate oncogenic stimuli and environment, become cancer stem cells (CSC).

There are several different theories on the evolution of cancer within an organ. It has long been recognized that exposure to certain toxins and chronic inflammatory states are known mechanisms of cancer development in several different organs such as the lung, liver and bladder [37–43]. These toxins are thought to cause specific mutations that lead to uncontrolled growth of tissues. While these mutations are acquired, inheritance of certain germline mutations like *RET* in multiple endocrine neoplasia (MEN), and *BRCA* for familial breast cancer can also produce tissue specific transformation as a reflection of a dysregulation of their physiologic roles. One of the best characterized examples of how the manifestation of a germ-line mutation within a progenitor cell pool results in carcinogenesis has been described for the familial form of colon cancer, familial adenomatous polyposis (FAP), in which a mutation within the adenomatous polyposis coli (APC) gene results in the adenoma-carcinoma sequence [44–45]. The *APC* gene is part of the Wnt signaling pathway destruction complex charged with maintaining low levels of active (non-phosphorylated) β-catenin levels when Wnt ligand mediated canonical signaling is activated. Disruption of *APC* mediation of the Wnt/β-catenin axis leads to increased intracellular â-catenin stability and subsequent target gene activation through nuclear de-repression of transcription.

In many stem cell compartments such as the hematopoietic and enteric systems Wnt/β-catenin signaling has the net effect of regulating progenitor cell proliferation and multipotency [46–49]. When an *APC* loss of function, or *β-CATENIN* gain of function mutation occurs within the crypt stem cell, control over proliferation of this compartment is lost leading to an overall expansion of these cells. These cells derive daughter cells that have already accumulated the mutation thus conferring a proliferative advantage to the mutated progenitors and their progeny over surrounding cells. The cumulative effect of the selective expansion of these self-renewing, mutated progenitor cells is a larger pool of cells susceptible to additional mutation and malignant transformation. An illustration of this progression can be seen in the intestine, where the phenotypic consequence of these mutations is a polyp or adenoma.

While significant advances have been made with respect to understanding the molecular biology of cancer for the purpose of developing new chemotherapeutics, the success rate of these treatments has remained low. It has recently been suggested that it is the cellular biology and not the molecular biology that belies our failure to produce effective drug regimens for cancer [50]. Two recent examples of the existence of a CSC phenotype in human breast and brain cancers are frequently cited as providing further important evidence for the hierarchical model of oncogenesis [31]. While this had been previously shown for acute myelogenous leukemia, evidence for this phenomenon in solid tumors was lacking. Al-Hajj implanted different human breast cancer specimens/cells into the mammary fat pads of NOD/SCID mice. Only a small subset of cells that were determined to be $CD44^{+}/CD24^{-/low}$ were capable of giving rise to new tumors with the same histopathology of the initial tumor. It is important to note that these cells represented a small proportion of the total breast cancer cells (1–3%). Similarly, in the case of brain

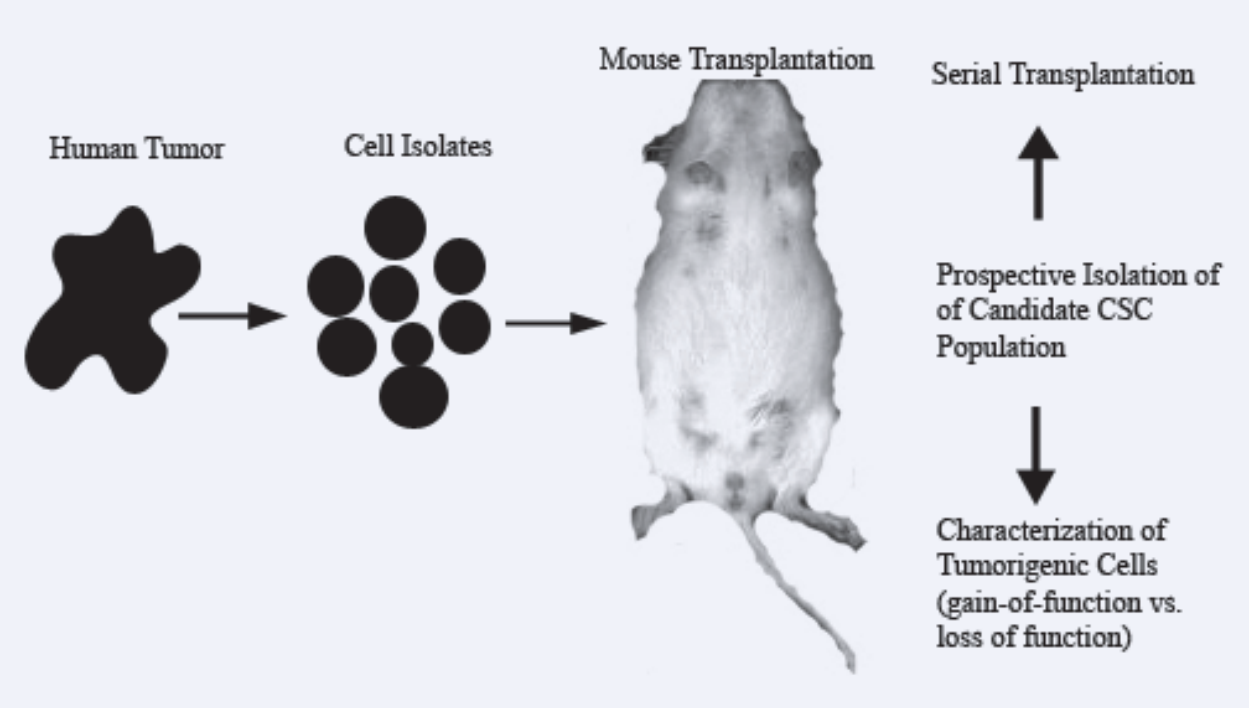

Fig. 5 Schema of testing human tumors for the cancer stem cell paradigm. Identification of tumor initiating cells occurs through a process of tumor cell isolate transplantation into immune-deficient mice. Following tumor growth, a limited candidate CSC population is prospectively isolated and assayed for both tumor initiation and propagation.

cancer, Singh *et al.* showed that a small fraction of $CD133^+$ cells isolated from human medulloblastoma and glioblastoma primary tumors were able to recapitulate the original tumor in NOD-SCID mice on serial transplantation (Fig. 5) [32, 33]. As few as 100 $CD133^+$ cells were capable of initiating the tumor within 12–24 weeks after injection into the frontal lobe of the mice while as many as 100,000 $CD133^-$ cells did not form tumors. CD133+ cells from human brain tumors had previously been shown to exhibit stem cell properties *in vitro* [33]. Taken collectively, these experiments lend strong support to the concept of a cancer stem cell (CSC) for solid tumors and hold considerable promise for the study of individual tumor characteristics for the development of better chemotherapeutics.

Signaling in tissue repair and regeneration

There is growing evidence that the pathogenesis of cancer involves the subversion of normal tissue repair mechanisms and stem cell signaling pathways [51, 52]. Strong support for this hypothesis stems from the observation that many cancers, such as hepatocellular or colorectal carcinoma, develop in the setting of chronic injury or inflammation [53–58]. As discussed above, normal tissue repair relies upon stem and progenitor cell proliferation for the replacement of lost tissue. In a state of chronic injury or inflammation, stem cells are under a continuous stimulus of proliferation. Two morphogenic signaling pathways, specifically Hedgehog (Hh) and Wingless (Wnt), serve to illustrate how pathways involved in stem cell proliferation during development, and regeneration have also been implicated in several different epithelial cancers [59–66].

Given the need for coordinated signaling for proliferation and differentiation during homeostasis it is not surprising that the involved signaling pathways are equally complex with numerous interactions both within and between their respective cascades. The Wnt proteins are a family of secreted glycoproteins that govern cell proliferation, migration, differentiation and polarity among cells during embryoge-

nesis. In adult organisms Wnt signaling has been shown to regulate hematopoietic, enteric and skin stem cell biology. There are nineteen Wnt proteins in mammals that bind with one of ten Frizzled receptors and/or with the LDL receptor-like proteins 5 and 6 (LRP5/6). Following binding, canonical Wnt signal transduction proceeds through the accumulation of cytoplasmic β-CATENIN. In the absence of Wnt ligand, β-CATENIN is held in a destruction complex composed of AXIN, APC and glycogen synthetase kinase-3â (GSK-3β). Phosphorylation of β-CATENIN by the destruction complex results in the ubiquitination and degradation of β-CATENIN. In the presence of Wnt protein binding, non-phosphorylated β-CATENIN accumulates, translocates to the nucleus and initiates target gene transcription by acting as a co-derepressor with TCF/LEF-1 transcription factors. Many of the β-CATENIN target genes like *C-MYC* and *CYCLIN-D1* regulate cell proliferation in a tissue and context dependent manner [67, 68]. Mutations in this pathway have been shown to be associated with several forms of hereditary and sporadic cancer both in the intestine and the liver [24, 64, 69, 70].

Wnt signaling is an important part of normal epithelial renewal within the small and large intestine. Enteric progenitor cells located within the crypts of Leiberkühn are stimulated to proliferate in response to Wnt signaling and resultant β-CATENIN-TCF4 complexes [66]. As these cells migrate from the crypts towards the surface epithelium, the distance from the Wnt source and resultant loss of canonical signaling through β-CATENIN commits these progenitor cells to terminally differentiate. As mentioned above, mutations within the APC tumor suppressor gene have been almost uniformly implicated in the pathogenesis of the inherited forms of colorectal carcinoma [71–73]. This gene product is an important part of the Wnt canonical signaling cascade and loss of its function leads to an accumulation of nuclear β-CATENIN with a consequent increase in proliferation [74–77]. These two findings provide the framework for how normal regenerative mechanisms can be subverted by tissues and tissue stem cells leading to malignant growth.

Hedgehog signaling has been shown to be an important antagonist to the Wnt pathway (Fig. 6) [62]. This protein, of which there are three types in humans (Sonic, Indian and Desert) acts through its receptors Patched and Smoothened by binding to Patched thereby allowing the release of Smoothened for activation of downstream target genes (*GLI*) [78]. Sonic Hedgehog has been implicated in postnatal and adult neurogenesis [79–81]. Machold and colleagues demonstrated its role in the maintenance of the stem cell niche within the mouse telencephalic forebrain subventricular zone [79]. Palma *et al.* also demonstrated the role of Hedgehog in the proliferation of the subventricular zone stem cells and the production of new olfactory interneurons in the postnatal and adult mouse [80, 81]. The authors report an important relationship between Hedgehog and epidermal growth factor (EGF) in the modulation of the stem cell proliferation. Both Hedgehog and Wnt signaling have been shown to be important in the regulation of stem cell self-renewal. Not surprisingly, increased activity of either of these pathways leads to increased proliferation of stem cells and, depending on the tissue, may also lead to cancer.

Notch, hedgehog and Wnt signaling in tumorigenesis

Many reports have shown a correlation between *β-CATENIN* gain of function mutations and hepatocellular carcinoma in mice and humans [23, 64]. This result notwithstanding, it is important to note that in a large proportion of these tumors it is the wild type β-CATENIN that accumulates, yet is not itself mutated. It is estimated that between 20% and 80% of HCCs show aberrant cellular accumulation of wild-type β-CATENIN [82]. Moreover, when we account for the absence of mutations in the APC complex or *AXIN* genes, it becomes clear that other mechanisms within the Wnt signaling cascade must account for the accumulation of β-CATENIN in these tumors. Cagatay *et al.* found an important association between P53 mutations and the aberrant accumulation of β-CATENIN in cancer cells [83]. However, given the low incidence of *P53* mutations, they cannot fully account for the overwhelming association between accumulation of β-CATENIN and HCC. By examining HCC tumors obtained from human males with hepatitis-related disease, Merle and colleagues were able to show that over-expression of the Frizzled transmembrane receptor 7 (FZD7) led to stabilized wild-type β-

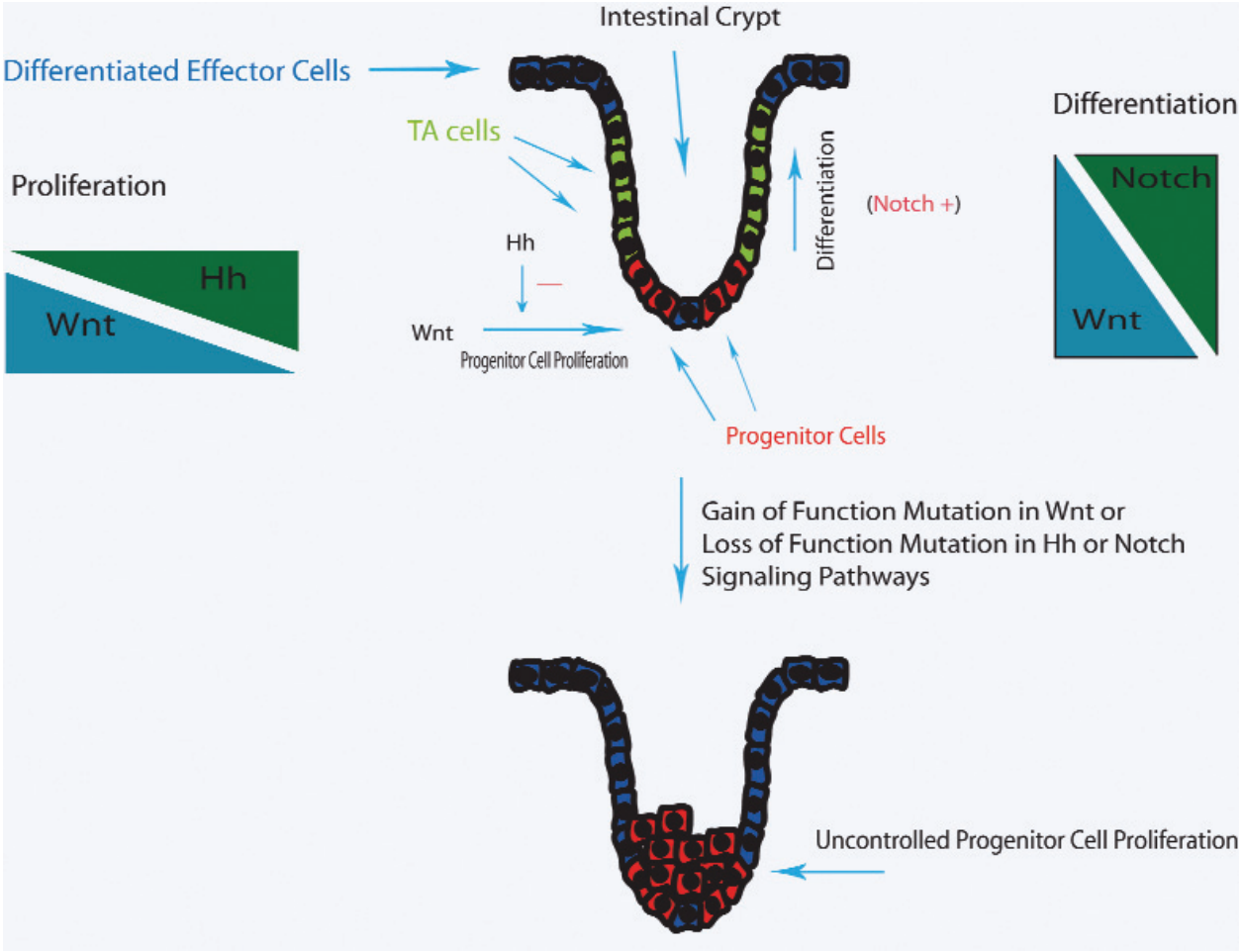

Fig. 6 Interplay of dominant morphogen pathways in stem cell maintenance and cancer development. Wnt, Hh and Notch signaling in the intestinal crypt controls progenitor cell proliferation and differentiation. The Wnt pathway leads to increased proliferation of progenitor cells which is inhibited by Hedgehog signaling. Notch signaling leads to increased differentiation of crypt progenitors. Loss of the careful balance between these signals leads to uncontrolled progenitor cell proliferation and may lead to malignancy.

CATENIN [82]. This protein was also over-expressed in four different human HCC cell lines all with increased β-CATENIN cellular accumulation. Of interest, the three cell lines with homozygous wild-type *β-CATENIN* alleles (Huh7, Focus and Hep3B) all showed strong nuclear localization of β-CATENIN, with over-expression of FZD7 and a strong correlation between this over-expression and increased *tcf* transcriptional activity [82, 84]. Even more compelling than these results was the finding that FZD7 was over-expressed in peritumorous areas, that is, in preneoplastic areas of the liver. This suggests that over-expression of FZD7 may represent an important early event in the pathogenesis of hepatocellular carcinoma. These experiments lead to the conclusion that there exists an important relationship between Wnt/β-catenin signaling and hepatocellular cancer.

Several signaling proteins are also responsible for controlling the differentiation of progenitor cells along specific pathways. Notch is an important signaling pathway throughout embryonic development and post natal tissue differentiation. This gene encodes a single transmembrane receptor that binds to one of five different ligands (Jagged 1 & 2 and Delta 1, 3 and 4). The ligands are also transmembrane proteins that allow association and communication between adjacent cells thereby controlling cell fates by cell-to-cell interactions. Binding of ligand to NOTCH results in proteolytic cleavage of its

intracellular domain (NICD) with subsequent translocation to the nucleus. There, NICD can bind to the transcriptional repressors CSL or CBF1 to permit transcription of a number of genes that regulate cell differentiation [84, 86]. In the intestine, the Notch signaling pathway has been shown *in vivo* to be an important inhibitor of differentiation. This was established using *Rosa*-NOTCH/*CRE*+ transgenic mice in which the intracellular domain of NOTCH-1 is over-expressed in intestinal cells. When compared against wild-type animals not over-expressing NOTCH, there is a marked difference in the number of proliferating cells [84]. These cells may represent a progenitor cell pool which is increased by the inhibition of cell differentiation in response to Notch signaling (Fig. 6). It is not surprising that Sonic Hedgehog, Notch and Wnt signaling pathways have all been implicated in oncogenesis.

The signaling pathways involving Hedgehog, Notch and Wnt are not completely independent (Fig. 6). In fact, there is an abundance of evidence that these pathways interact to control epithelial differentiation in several tissues [88–90]. Van den Brink and colleagues studied the interaction between Hedgehog and Wnt pathways in colonic epithelial cell differentiation and showed Indian hedgehog (IHh) to have an antagonistic effect on Wnt signaling in colonic epithelium [62]. While Wnt signaling maintains the colonic epithelial progenitor cell phenotype, Hh signaling appears to be important for progenitor cell differentiation within the colon leading to increased differentiation and decreased proliferation [66, 91]. An examination of β-catenin-TCF signaling in DLD-1 colon cancer cells showed that IHh signaling completely inhibited this pathway. The converse was also found to be true in sporadic colonic adenomatous polyps and polyps from patients with FAP where Wnt signaling was found to inhibit IHh signaling. No dysplastic or cancer cells were found to express hedgehog. Thus, mutations of *APC* which lead to constitutive activation of the Wnt pathway lead to inhibition of the hedgehog pathway, increased proliferation of colonic epithelial precursor cells and malignant growth (Fig. 6).

Hedgehog signaling has also been implicated in the development of medulloblastoma, a common cerebellar malignancy of childhood. Hedgehog normally mediates its cellular response through its receptor Patched and the seven-transmembrane signaling protein Smoothened [92]. Mouse medulloblastoma tumor cells *in vitro* and *in vivo* treated with cyclopamine, a known inhibitor of hedgehog signaling through binding of Smoothened, showed blocked proliferation of tumor cells with evidence of increased differentiation. Moreover, treatment with cyclopamine led to regression of allograft tumors propagated in nude mice [63, 88]. This inhibition of proliferation through cyclopamine treatment was subsequently relieved by transfection of cells with *GLI*. GLI is a known downstream activator of Hedgehog [93, 94]. Interestingly, neither glioblastoma nor ependymoma cells were found to be responsive to cyclopamine. These experiments suggest that hedgehog signaling is an important contributor to the development and maintenance of medulloblastoma. It is generally accepted that mutation within the external germ layer (EGL) leading to uncontrolled proliferation of progenitor cells is a known cause of medulloblastoma [95]. The Sonic Hedgehog (SHh) pathway has several downstream targets that may be involved in its pathogenesis. Specifically, the polycomb group gene Bmi1 has been shown to be overexpressed in medulloblastoma cell lines and human tumors [96]. Expression of this gene was shown to be increased in cerebellar granule cell cultures following SHh treatment suggesting it is a downstream target of SHh signaling. Furthermore, treatment of BMI-1-/- granule cells with SHh showed decreased proliferation in a BrdU incorporation assay as compared to *BMI-1*+/+ cells. This provides further evidence of the downstream activation of *BMI-1* in the SHh pathway and its role in promoting proliferation.

The role of BMI-1 and Sonic Hedgehog in promoting progenitor cell self-renewal and proliferation was also recently shown in normal hematopoietic and leukemic stem cells [97–100]. In these experiments, *BMI-1*-/- leukemic cells were unable to recapitulate AML in secondary recipients [101]. BMI-1 deficient cells were found to have reduced proliferative abilities. The ability to induce acute myelogenous leukemia in recipients of *BMI-1* -/- cells was able to be restored with a retroviral construct expressing BMI-1. *BMI-1* -/- cells were also found to have an increased proportion of apoptotic cells. This was consistent with the findings of Leung and colleagues in medulloblastoma who suggested that the inability of progenitor cells to respond to proliferative stimuli leads to programmed cell death [95]. Clearly, the role of BMI-1 and SHh signaling

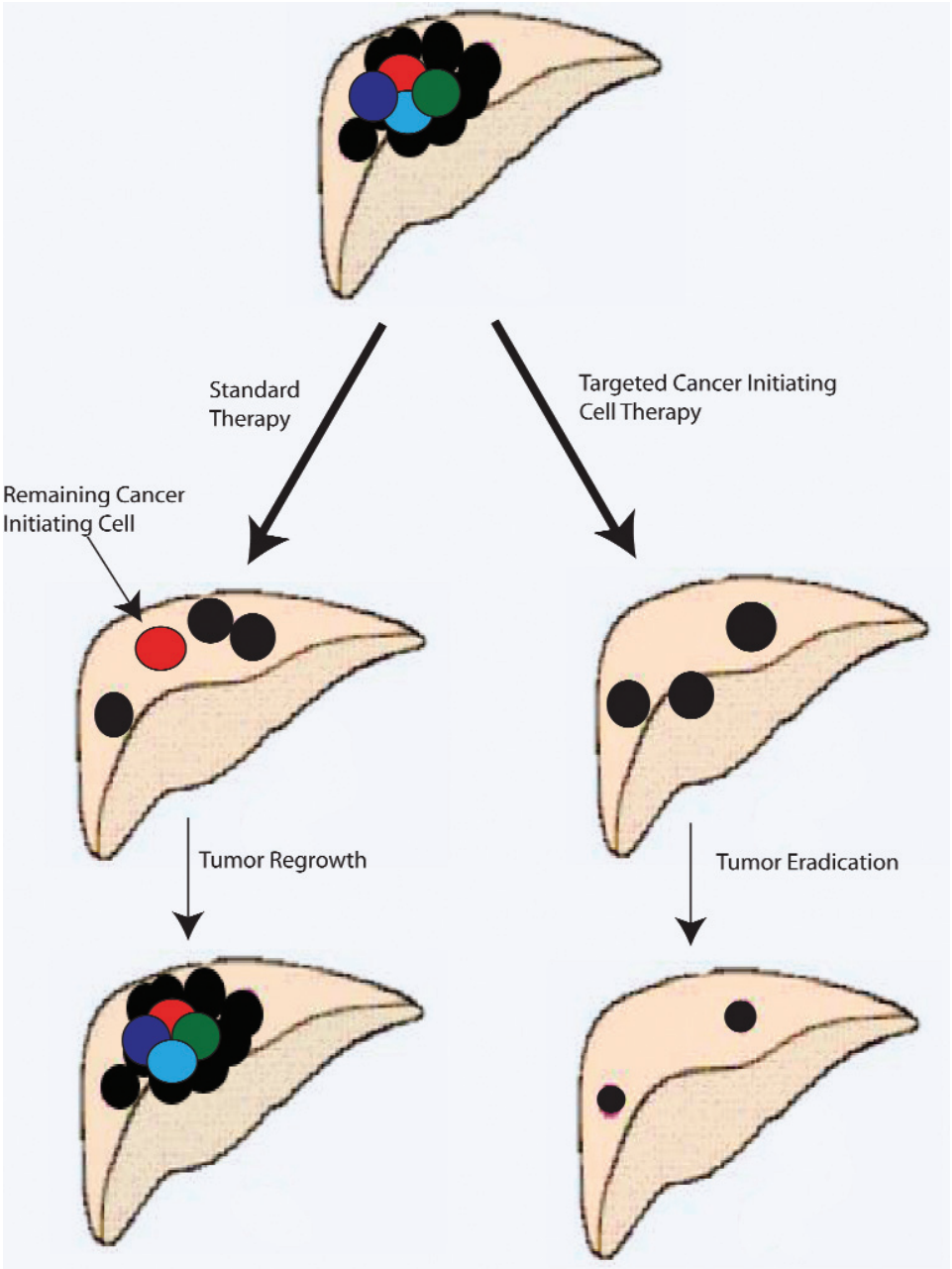

Fig. 7 New paradigm of cancer stem cells targeted therapies. Cancer stem cells are often resistant to chemotherapy and may persist after resection. Subsequent growth of these cells is sufficient to allow re-growth of the tumor. In the new cancer stem cell targeted treatment model, elimination of these cells may prevent tumor recurrence.

in the generation of these tumors must be elaborated in order to develop better treatment strategies.

Perspectives: reconciling cellular and molecular biology for better cancer treatments

The ultimate goal of cancer research is the complete understanding of the mechanisms of disease initiation and progression for the development of more effective treatments. If we are to succeed in this endeavor a successful bridging between the cellular biology and the molecular biology of cancer must take place. Researchers have already taken important steps toward this end by prospectively isolating cancer initiating cells in solid tumors. This will facilitate the study of the biological behavior of patient tumors as a function of the CSC paradigm thus allowing a more targeted approach to therapy of individual tumors (Fig. 7). In terms of cell signaling, several advances have been

made over the years to contribute to the current advanced knowledge and understanding of the importance of the Hedgehog, Wnt and Notch pathways in carcinogenesis. These discoveries notwithstanding, there remains a need to learn the specific signals that trigger the cancer initiating stem cell to transform for each specific tissue. It is likely that we will find that the tumor's dependence on a specific transforming mutation may also remain dependent on that same mutation for the tumor's propagation. From the complexity of signaling involved in carcinogenesis, it is clear that each cancer is unique in the set of signals and group of cells involved in its development.

Therefore, if we are to develop newer more effective treatments for cancer, several obstacles must first be overcome. First, we need to determine the cancer initiating cell in the various forms of solid organ cancer. Identifying these cells will allow us to perform gene and protein profiling studies to determine how the expression patterns of these cells differ from other cancerous cells within the same tissue. Since cancer stem cells are believed to be more resistant to our current chemotherapeutics, allowing them to persist and regenerate tumors, understanding these different expression patterns will be of paramount importance in producing more effective treatments. Second, we will need to learn the early events involved in the process of malignant transformation of these cancer initiating cells. This will preclude the need for palliative surgery for advanced cases as we would be able to stop cancers before they have an opportunity to become invasive and widely metastatic. Even more speculative, this may lead to a cancer vaccine approach to prohibit these critical signaling pathways from initiating transformation in those cell populations that we deem most susceptible. Finally, we will need to develop animal models of these cancers to allow the recapitulation of tumors from a single or a few cancer-initiating cells. This will permit the *in vivo* study of the natural history of individual tumors and further facilitate the process of individualized patient care. With these goals accomplished, we will be able to advance into the next generation of cancer treatment and better patient care.

Acknowledgements

Scott V Perryman is the recipient of a Daland Clinical Fellowship from the American Philosophical Society. This work was also supported in part by the NIH DE015368, and a Harmon Clinical Scholars Fellowship (KGS).

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