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REVIEW

Stem cells and cancer: a deadly mix

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Abstract Stem cells and cancer are inextricably linked; the process of carcinogenesis initially affects normal stem cells or their closely related progenitors and then, at some point, neoplastic stem cells are generated that propagate and ultimately maintain the process. Many, if not all, cancers contain a minority population of self-renewing stem cells, "cancer stem cells", that are entirely responsible for sustaining the tumour and for giving rise to proliferating but progressively differentiating cells that contribute to the cellular heterogeneity typical of many solid tumours. Thus, the bulk of the tumour is often not the clinical problem, and so the identification of cancer stem cells and the factors that regulate their behaviour are likely to have an enormous bearing on the way that we treat neoplastic disease in the future. This review summarises (1) our knowledge of the origins of some cancers from normal stem cells and (2) the evidence for the existence of cancer stem cells; it also illustrates some of the stem cell renewal pathways that are frequently aberrant in cancer and that may represent druggable targets.

Keywords Carcinogenesis · Cancer stem cells · Clonogenicity · Stem cell markers · Haematopoietic stem cells · Side population · Signalling pathways

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Normal adult stem cells

Cells with the key properties of adult stem cells, namely indefinite self-renewal and multilineage potential, were first recognised in the bone marrow (BM). Such cells were first described experimentally back in 1961 as cells that gave rise to multilineage haematopoietic colonies in the spleen (colony-forming units; Till and McCulloch 1961). When cell migration occurs in a tissue, stem cells are at the beginning of the flux. In particular, when cell flux is unidirectional, e.g. in the epidermis, stem cells are at the beginning of the cell flux in the basal layer, with cells being shed at the surface. To achieve self-renewal, on average, each stem cell division gives rise to one replacement stem cell and one transit amplifying cell (TAC) by asymmetric cell division. Thus, the probability (P) of self-renewal is 0.5 and any upward shift in P will lead to a rapid escalation in stem cell numbers, a likely event in many cancers (Clevers 2005). Indeed, disruption of asymmetric neuroblast cell division in *Drosophila melanogaster* leads to lethal expansive tumour-like lesions when such cells are transplanted to adult hosts (Caussinus and Gonzalez 2005).

Stem cells are located in regulated environments called niches and, in a tissue such as the small intestine, the niche is found close to the crypt base at the origin of the flux. Interactions with the niche cells are crucial to the self-renewal process and the controlling factors are rapidly being elucidated. For example, in the gut, Wnt signalling is a requirement for stem cell homeostasis (Sancho et al. 2004).

Stem cells are believed to be proliferatively quiescent in comparison with TACs. Teleologically, it would seem prudent to limit stem cell replication rates because of the error-prone nature of DNA synthesis. If stem cells do divide infrequently, then stem cells that incorporate DNA synthesis labels such as tritiated thymidine or bromodeoxyuridine (BrdU) would remain "labelled" for longer than TACs

whose more rapid cycling would soon dilute the label below detection levels. Thus, the identification of label-retaining cells (LRCs) might be a useful stem cell marker in some cases (Braun and Watt 2004; Alison et al. 2006a, b) but, in the haematopoietic system where haematopoietic stem cells (HSCs) can be definitively identified, label retention would seem a poor marker of HSCs (Kiel et al. 2007). Some stem cells appear to have devised a strategy for maintaining genome integrity; another cause of "label retention". Termed the "immortal strand" hypothesis by John Cairns (Cairns 1975), newly forming stem cells designate one of the two strands of DNA in each chromosome as a template strand, such that, in each round of DNA synthesis when both strands of DNA are copied, only the template strand and its copy are allocated to the daughter cell that remains a stem cell. Thus, any errors in replication are readily transferred (within one generation) to TACs that are soon lost from the population. Such a mechanism will produce LRCs after injection of DNA labels when stem cells are being formed, although validation of the hypothesis requires the observation that LRCs go through further rounds of DNA synthesis, while still segregating the immortal DNA strands from sister chromatids into the daughter cells that remain as stem cells; this has been demonstrated in the small intestine and breast (Potten et al. 2002; Smith 2005). The existence of LRCs during normal stem cell renewal is based upon the random segregation of sister chromatids; however, this is incompatible with the "immortal strand hypothesis", which predicts the loss of a newly incorporated DNA synthesis marker within two cycles (Rando 2007). Recently, an alternative epigenetic explanation for asymmetric cell division has been proffered, termed the "silent sister hypothesis", proposing that the daughter cell remaining as a stem cell selectively retains chromatids with active stem cell genes, with the loss of stem cell properties in the cell that inherits the opposite "silent" sister chromatids (Lansdorp 2007).

Considerable effort is being expended in the search for "markers" of stem cells, with every expectation that many of the molecules expressed by normal stem cells will also be found in their malignant counterparts. Collectively, these molecules appear to be involved in maintaining "stemness" (transcription factors, such as Oct-4 and Nanog), ensuring adhesion to the niche and being involved in cytoprotection (Alison et al. 2006a, b). For example, in the human epidermis, superior colony-forming ability has been found from cells selected on the basis of expression of the hemidesmosomal integrin α6, which partners β4 to attach cells to the basement membrane component, laminin V (Webb et al. 2004), whereas Jones and Watt (1993) have reported the selection of basal keratinocytes based upon the high expression of the β1-integrin enriched for colony-forming ability. Likewise, in the murine mammary gland, either the β-1 integrin (CD29) or the α-6 integrin (CD49f) has been used to isolate multipotential stem cells, each capable of generating entire mammary glands after in vivo transplantation (Shackleton et al. 2006; Stingl et al. 2006).

Another strategy adopted by stem cells appears to confer resistance to xenobiotics, including many of the drugs used today to treat cancer. For example, HSCs have high levels of aldehyde dehydrogenase (ALDH), a detoxifying enzyme that confers resistance to alkylating agents such as cyclophosphamide (Storms et al. 1999). Indeed, in acute myeloid leukaemia (AML), a subpopulation of $CD34^{+}$ cells expressing high levels of ALDH defines a poor prognosis group (Cheung et al. 2007); this population is highly enriched for tumour-initiating cells in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. In the bronchiolar epithelium, stem cells appear to be rare pollutant-resistant cells linked to a deficiency in the phase I drug metabolising enzyme CYP450 2F2 (Giangreco et al. 2004). In the liver too, progenitor cells can expand, despite exposure to pyrrolizidine alkaloids, because of a similar deficiency in cytochrome P450 (Vig et al. 2006).

Perhaps the most common defence mechanism adopted by stem cells relates to their high expression of members of the ATP-binding cassette (ABC) superfamily of membrane transporters. Such proteins are characterised by expression of an ABC region functioning to hydrolyse ATP to support energy-dependent substrate efflux against steep concentration gradients across membranes, principally from the intracellular cytoplasm to the extracellular space. This observation was exploited by Goodell et al. (1996) who first reported the isolation of HSCs based on their ability to efflux a fluorescent dye. Like the activity of the P-glycoprotein (encoded by the *mdr1* gene), this activity is inhibited by verapamil. If cells are subjected to Hoechst 33342 dye staining and fluorescence-activated cell-sorting analysis, then those that actively efflux the Hoechst dye appear as a distinct population of cells on the side of the profile, hence the name the "side population" (SP) was given to these cells. Many studies suggest that the SP phenotype in rodent and humans tissues is largely determined by the expression of a protein known as the ABCG2 transporter (ABC subfamily G member 2, also known as breast cancer resistance protein) and that the SP fraction equates with the stem cell population in some normal tissues (Alison 2003) and tumours (Hirschmann-Jax et al. 2004, 2005). However, not all SP cells are stem cells; in the mouse hair follicle, the SP cells are found beneath the "label-retaining" bulge and thus correspond to TACs (Triel et al. 2004), whereas in the human epidermis and the murine haematopoietic system, the SP phenotype does not specify all stem cells (Triel et al. 2004; Morita et al. 2006). The over-expression of ABC transporters by cancer stem cells (CSCs) could, of course, contribute to relapsed and drug-resistant cancers because of their efficient drug-effluxing ability.

As discussed later, an increasingly adopted marker for the enrichment of CSCs in many tumours is a molecule called prominin-1 or CD133. CD133 is a pentaspan membrane protein; in neuroepithelial cells, it may be involved in regulating asymmetric stem cell divisions (Marzesco et al. 2005).

What are the founder cells of cancer?

For many years, it has been apparent that stem cells feature in processes as diverse as wound healing, metaplasia and cancer. Two-stage models of skin cancer in rodents carried out in the 1950s strongly suggested that cancers had their origins in long-lived epidermal stem cells but the idea that cancers themselves might have malignant CSCs is only just gaining widespread acceptance, despite the fact that Hamburger and Salmon (1977), using colony formation in soft agar as a surrogate stem cell assay, found that, for many human tumours, only 1 in a 1000 to 1 in 5000 cells was able to form a macroscopic colony.

Cancer could arise from the dedifferentiation of mature cells that have retained the ability to divide or it could result from the "maturation arrest" of immature stem cells (Sell and Pierce 1994). The idea of "blocked ontogeny" has gained wide acceptance and we now believe that the arrested differentiation of tissue-based stem cells or their immediate progenitors is closely linked to the development of not only teratocarcinomas and haematological malignancies, but also carcinomas. Some of the most frequent cancers occur in tissues with a high cell turnover, such as the skin and the epithelial lining of the gastrointestinal tract. It is argued, not unreasonably in our view, that the stem cells in these tissues are the only cells with sufficient lifespan to acquire the requisite number of genetic abnormalities for malignant transformation. Additionally, with a self-renewal mechanism already in place, seemingly fewer alterations are required to change normal stem cells into CSCs. In the now classical two-stage model of mouse skin carcinogenesis, severely delaying the interval between 7,12-dimethylbenz(a)anthracene initiation and the application of the phorbol ester promoter had no bearing on subsequent tumour yield, strongly suggestive of an origin in a long-lived cell, namely an epidermal stem cell (Berenblum and Shubik 1949).

In many haematological malignancies, an origin from normal HSCs is indicated by the finding that one of the oncogenic alterations (presumably the earliest) can also be found in a variety of haematopoietic progenitor and differentiated cell types in patients in remission, again suggestive of an origin in a multipotential stem cell that subsequently accrues further mutations that lead to malignancy. This can be observed in AML in which the balanced translocation t(8;21) leads to the fusion transcript *AML-ETO* being found in many non-leukaemic cells (Miyamoto et al. 2000). Likewise, in chronic myeloid leukaemia (CML) in which 95% of affected individuals have the Philadelphia chromosome, the *BCR-ABL* fusion transcript can also be found in otherwise normal mature blood cells (Cobaleda et al. 2000).

A stem cell origin of cancer is often inferred from the location of the tumour or its dysplastic antecedents but this does not preclude an origin from a more committed progenitor cell in the same location. For example, in the human brain, gliomas are frequently found in a periventricular location, an observation first made in the human brain some 60 years ago, and experimental brain tumours induced by ethyl nitrosurea (ENU) are usually found in the subventricular zone, a major germinative area of the central nervous system (CNS; reviewed by Sanai et al. 2005; Vescovi et al. 2006). The heterogeneity of gliomas also indicates an origin from a multipotent stem cell. In the mouse lung, the bronchioalveolar duct junction is a major stem cell zone for the airway; ras-induced adenocarcinomas arise in this area containing clusters of cells with the same stem cell characteristics (Kim et al. 2005).

In the liver, a variety of intrahepatic stem cells can give rise to hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC; Alison and Lovell 2005). Many liver tumours arise in a cirrhotic setting at a time when hepatocyte senescence triggers a reserve stem cell compartment to give rise to bipotential hepatic progenitor cells (HPCs); the finding that many liver tumours have features of both HCC and CC, combined with the presence of numerous HPCs, is not inconsistent with an origin from HPCs or their antecedents. Indeed, gene expression profiling has identified a subset of HCCs with a poor prognosis and a profile consistent with an origin from HPCs (Lee et al. 2006).

Chronic inflammation is seen as a major pathogenic mechanism for the promotion of primary liver tumours, where sustained cell proliferation in a milieu rich in inflammatory cells, growth factors and DNA-damaging agents (reactive oxygen and nitrogen species produced to fight infection) will lead to permanent genetic changes in proliferating cells. The upregulation of the transcription factor NF-κB in transformed hepatocytes, stimulated by the paracrine action of tumour necrosis factor-α from neighbouring endothelia and inflammatory cells, appears critical for tumour progression in some cases, given the mitogenic and anti-apoptotic properties of proteins encoded by many of the target genes of NF-κB (iNOS, COX-2 and Bcl-X_L; Pikarsky et al. 2004). In addition, with regard to hepatitis B virus, viral DNA may integrate into host DNA, activating proto-oncogenes or virally encoded proteins (e.g. HBx and hepatitis B spliced protein) may interact with several key regulatory proteins (Kremsdorf et al. 2006). The tumori-

genicity of hepatitis C virus has hitherto largely been attributed to chronic inflammation but, here also, viral gene products may modulate potentially oncogenic pathways such as Wnt signalling (Levrero 2006).

The cells that line the gastrointestinal tract are amongst the most rapidly proliferating cells in the body, with differentiated cells undergoing continual replacement. The rapid turnover of the gastrointestinal epithelium means that differentiating cells are shed into the lumen and replaced every few days and thus do not have a sufficient lifespan to gather the multiple genetic defects required for malignant transformation. Therefore, the perpetual stem cell has long been considered the target of carcinogenic mutations. In the colon, inspection of the earliest dysplastic lesions suggests an origin from a mutated, basally situated, stem cell that "hitchhikes" its way to clonal dominance in a crypt of multiple stem cells through a process known as "niche succession" (Calabrese et al. 2004) to form a monocryptal adenoma (Fig. 1). Although it is a widely held view that tumours are clonal populations, studies of early familial adenomas in both patients with familial adenomatous polyposis (FAP) and those in the mouse model ($Apc^{Min/+}$) suggest that the majority of nascent tumours are indeed polyclonal, requiring short-range interactions between adjacent initiated crypts: "collision" tumours as a cause of this apparent polyclonality has been ruled out by genetically manipulating the $Apc^{Min/+}$ mouse to have far fewer tumours than normal (Thliveris et al. 2005). Park et al. (1995) used ENU to induce mutations in the X-linked gene for glucose-6-phosphate dehydrogenase (G6PD) to demonstrate the expansion of a mutated clone within the crypt. *G6PD* gene mutation resulted in the loss of enzyme activity in the affected cells. After ENU treatment, Park et al. (1995) initially observed crypts that were only partially positive for G6PD but that eventually disappeared with the contemporaneous emergence of fully mutated crypts (monoclonal conversion or crypt purification): these eventually gave rise to patches of crypts that failed to express G6PD. More recently, Taylor et al. (2003) have used mitochondrial DNA mutations in colonic crypt cells to demonstrate the presence of partially mutated crypts in the human colon. They have observed that human colonic crypt cells accumulate sufficient mitochondrial DNA (mtDNA) mutations with age to cause a biochemical defect in the mtDNA-encoded subunits of cytochrome c oxidase (COX). Normal colonic tissue shows numerous completely COX-deficient crypts, but also a few partially stained crypts. Serial sections of these partial crypts have allowed Taylor et al. (2003) to reconstruct three-dimensional images of the crypt, revealing a ribbon of COX-negative cells extending from the base of the crypt to the top. Each ribbon of mutated COX-negative cells appears to be the progeny of one of the small number of stem cells in the niche, whereas the partially negative crypts are likely to be intermediate steps in the expansion of the mutated clone with eventual formation of a completely clonal COX-deficient crypt.

If niche succession is a way by which a single stem cell line can "hitchhike" its way to clonal dominance in a single crypt, then how does a mutated stem cell line expand into adjacent tissue? As described above, clonality experiments in both mice and humans have shown the clustering of mutated, phenotypically similar crypts together in patches. A process called "crypt fission", whereby crypts undergo basal bifurcation followed by longitudinal division with the ultimate formation of two daughter crypts, is thought to be responsible for the clustering of apparently related crypts and is undoubtedly the mechanism by which dysplastic crypts multiply to form microadenomas or dysplastic aberrant crypt foci (ACF; Wasan et al. 1998; see Fig. 1). ACF are morphologically and genetically distinct lesions that are the precursors of adenomas and cancers (Takayama et al. 1998).

The expansion of a mutated clone from a single cell to form a small adenoma is contentious, with two main theories: the top down and bottom up models (Fig. 2). The top down model was based upon the frequent observation of dysplastic cells solely at the luminal surface of the crypts (Shih et al. 2001), together with the apparent

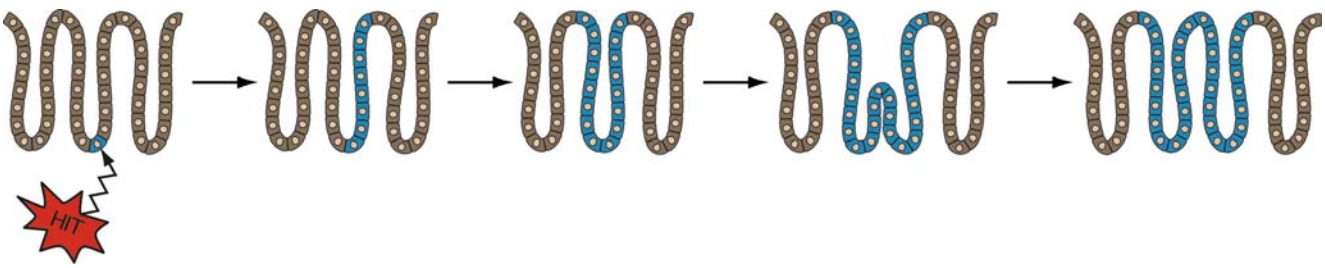

Fig. 1 Model for the development of early colorectal cancer. Mutation occurs in a stem cell located near the base of the crypt and mutated cell progeny occupy part of the crypt (*brown* normal colonocytes, *blue* mutated colonocytes). Through a stochastic process (called "niche succession" or "monoclonal conversion"), the affected crypt becomes wholly occupied by dysplastic cells: the monocryptal adenoma. Further expansion can occur by the dysplastic crypt undergoing crypt fission and budding, leading to an oligocryptal adenoma (aberrant crypt focus)

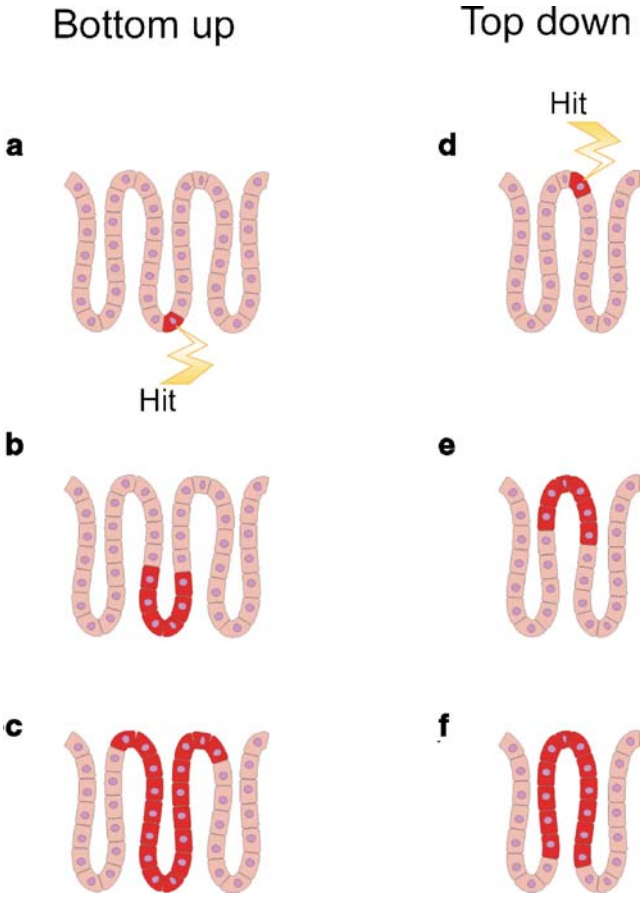

Fig. 2 "Top down" or "bottom up" growth of colorectal adenomas? *Bottom up* The stem cell, located in the crypt base, undergoes *APC* mutation (**a**). The mutated cell proliferates (**b**) and spreads to the top of a crypt to form a monocryptal adenoma (**c**). Initial further expansion is by crypt fission; based on Preston et al. (2003). *Top down* The initial transformation event occurs in a cell in the intercryptal zone (**d**) and then spreads laterally and downwards (**e**) eventually filling the whole crypt (**f**); adapted from Shih et al. (2001)

retrograde migration of adenomatous cells from the surface to the base of the crypt. Only these upper crypt cells show prominent proliferative activity and the nuclear localisation of β-catenin. These observations are not easily reconciled with the conventional view of the stem cell origin of cancer, and Shih et al. (2001) have proposed two possible explanations to explain their findings. First, they have considered a relocation of the stem cell area to the intercryptal zone and, second, they suggest that a mutated stem cell migrates from the base of the crypt to the luminal surface before expanding laterally and downwards. The bottom up model involves the recognition of the earliest lesion in tumour development, the "monocryptal adenoma", in which the dysplastic cells occupy an entire single crypt. Analysis of tiny (<3 mm) adenomas in FAP patients has shown increased proliferative activity and nuclear β-catenin translocation in morphologically dysplastic cells from the crypt base to the luminal surface (Preston et al. 2003). Additionally, a sharp cut-off occurs between the dysplastic surface epithelium with nuclear β-catenin expression and the normal mucosa in a neighbouring unaffected crypt. The observation of an increased asymmetrical crypt fission index in adenomatous tissue has led the researchers to propose the bottom up model: an abnormal stem cell clone with a growth advantage expands from the stem cell niche at the crypt base to fill an entire crypt. Thereafter, initial spread is by crypt fission to form an ACF, with top down spread undoubtedly occurring in slightly larger lesions (Preston et al. 2003).

BM-derived cells (BMDCs) can, under certain circumstances, cross their normal lineage boundaries and differentiate into non-haematopoietic tissue, including epithelia, because of so-called "stem cell plasticity" (Alison et al. 2006a, b). Although an interesting observation, the relatively low levels of conversion of BMDCs to epithelia does not suggest an axis of regeneration of any great therapeutic or clinical significance. However, Houghton et al. (2004) have made the profound observation that, under special circumstances, BMDCs might be the founder cells for gastrointestinal cancer. They have shown, in a murine model of gastric cancer, that BMDCs home to and repopulate the gastric mucosa and, over time, contribute to metaplasia, dysplasia and cancer in response to chronic infection with *Helicobacter felis*. BM-derived gastric glands are first seen 20 weeks after chronic infection commences, leading to a final replacement of 90% of the gastric mucosa with BMDCs after 1 year. Upon progression to epithelial dysplasia and gastric adenocarcinoma, the majority of the dysplastic glands are of BM origin, most likely from mesenchymal stem cells. The key to this observation is that chronic inflammation probably ablates the stem cell niches of the indigenous gastric glands; the vacant niches are then occupied by BMDCs that subsequently behave as gastric gland stem cells (Fig. 3).

Whereas most CSCs probably originate from transformed normal stem cells, they could also have their origins from more committed cells caused through, for example, reactivation of self-renewal mechanisms. Enabling stem cell-like features to occur in more mature cells may permit a CSC to emerge from a cell that was not originally a tissue stem cell (Passegue et al. 2003). For example, when HSCs and myeloid progenitor cells are transduced with a leukemogenic *MLL-ENL* fusion gene and the cells are transplanted in vivo, AML arises from both populations with the same latency (Cozzio et al. 2003). Moreover, their immunophenotype and gene expression profiles suggest maturation arrest at identical stages of the differentiation pathway. Likewise, the *MOZ-TIF2* oncogene can confer leukaemic stem cell properties (can be continuously propagated in liquid culture and result in AML after transplantation) upon granulocyte-monocyte progenitors

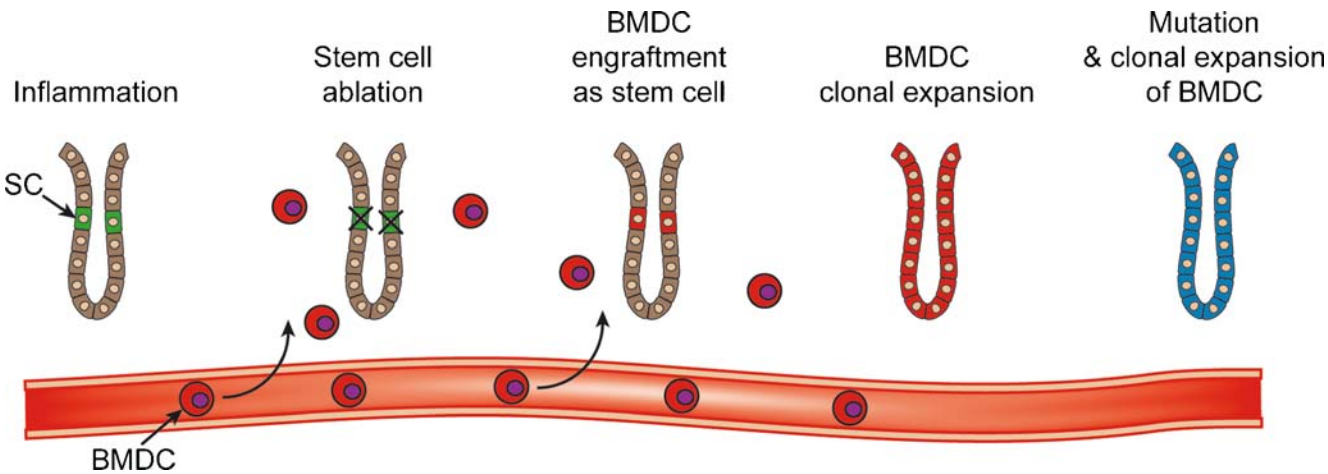

Fig. 3 A new paradigm of epithelial cancer development in the gastric gland. Continued inflammation and tissue damage leads to eradication of the indigenous stem cell compartment and its replacement by bone-marrow-derived cells (*BMDC*), whose progeny subsequently repopulate the whole gland. Mutation in a BMDC engrafted as a stem cell can then lead to a dysplastic gland and subsequent gastric cancer (*brown* indigenous normal epithelial cells, *green* indigenous stem cells, *red* BMDCs, *blue* mutated BMDCs)

(GMPs; Huntly et al. 2004). Interestingly, the *BCR-ABL* oncogene is not able to do this, indicating a hierarchy of leukaemia-associated proto-oncogenes. Introduction of the MLL-AF9 fusion protein can also generate leukaemic stem cells from GMPs (Krivtsov et al. 2006); furthermore, these cells maintain the gene expression profile of normal GMPs with the exception of a subset of self-renewal genes found in normal HSCs, indicating that widespread reprogramming is not required for such a switch.

The stem cell profile may also change during the course of a disease. In patients with CML in blast crisis, GMPs can become self-renewing CSCs, a change associated with Wnt pathway activation (Jamieson et al. 2004).

Tumour-initiating cells: CSCs

It is widely accepted that not all cells within a tumour are equally able to form tumours. Rather, the tumorigenic capability seems to be exclusive to a small minority of tumour cells, the so-called CSCs, that exhibit stem cell characteristics, particularly the ability to self-renew and to give rise to a hierarchy of progenitor and differentiated cells, albeit in a disorganised manner that gives rise to more CSCs. Operationally at present, we regard CSCs as prospectively purified cells that are more tumorigenic than the bulk or the marker-negative tumour population in a suitable tumour development assay, e.g. after transplantation to NOD/SCID mice. More stringently, a CSC should be a cell that can reconstitute, in a recipient animal, a tumour that is identical to the original tumour in the patient and that can then be serially xenotransplanted indefinitely. At present, most putative CSCs are identified by their tumour-initiating ability.

The first connection between tissue development and tumour formation was made in 1855 by Rudolph Virchow. Based on histological similarities between the developing fetus and cancers such as teratocarcinoma, he suggested his "embryonal-rest hypothesis" of cancer, believing that tumours resulted from the activation of dormant residual embryonic tissue (Virchow 1855). This hypothesis is supported by the finding that, in certain cancers, clusters of abnormal retained embryonic cells and cancer-associated mutations can be detected prenatally or at birth (Beckwith et al. 1990; Wiemels 1999).

In an ethically controversial experiment in which only a few tumour cells from patients with disseminated malignancies showed tumour-initiating capacity when injected back into the same patients, Brunschwig et al. (1965) demonstrated, for the first time, that tumour cells seemed to be hierarchically organised. Other evidence for a hierarchical organisation within a tumour came from the observation that only 1%–4% of lymphoma cells were able to form colonies in vitro or generate spleen colonies when transplanted in vivo (Bruce and Van der Gaag 1963). Likewise, studies of human tumours revealed clonogenic potentials in vitro in the range of 0.0001%–1% (Hamburger and Salmon 1977).

With the development of NOD/SCID mice for the xenografting of human tumours came the first good in vivo evidence for the existence of CSCs (Bonnet and Dick 1997). Only a small subset of AML cells was shown to be capable of producing leukaemic progenitors and leukaemic blasts upon transplantation into immunodeficient mice, resulting in a disease phenotype identical to the donor (Bonnet and Dick 1997): the putative CSCs had the same phenotype, $CD34^{+}CD38^{-}$, as described for primitive HSCs. Since the first account of CSCs in leukaemia, they have also been demonstrated in various solid tumours. In the

breast, the existence of stem cells has been inferred by the clonogenicity (ability to form mammospheres) and multipotentiality of a subpopulation of murine mammary cells (Dontu et al. 2003; Al-Hajj et al. 2003). In breast cancer, a subpopulation of cells with the surface antigen expression pattern of $ESA^{+}CD44^{+}CD24^{-/low}$ is able to form tumours when implanted in limiting dilution into immunodeficient mice and to self-renew as shown by serial transplantation (Al-Hajj et al. 2003). These same tumour-initiating cells have been demonstrated to be capable of in vitro mammosphere generation (Ponti et al. 2005). By using mammosphere formation in non-adherent culture as a surrogate assay, presumptive CSCs have also been identified in a premalignant stage of breast cancer, namely ductal carcinoma in situ (Farnie et al. 2002).

In human pancreatic cancer, a cell population with a phenotype closely related to breast CSCs, i.e. $CD44^{+}CD24^{+}ESA^{+}$, is greatly enriched for tumour-initiating ability (Li et al. 2007): making up only 0.2%–0.8% of all cells, as few as 100 of these cells produce tumours in 50% of mice. In ovarian cancer, CD44, together with the expression of c-erbB1, c-Met, Nanog and Oct-4, has been used to enrich for clonogenic and tumour-initiating cells (Bapat et al. 2005). CD44 is also expressed by a minority (<10%) population of cells in head and neck squamous carcinoma, cells that also expressed the basal cytokeratins, CK5/14. Can these be considered as presumptive CSCs (Prince et al. 2007)? CD44 also features in prostate cancer; a subpopulation with the signature $CD44^{+}\alpha2\beta1^{high}CD133^{+}$ has no correlation with tumour grade but is substantially enriched for in vitro colony-forming ability (Collins et al. 2005). CD44 has been confirmed as a useful prostate CSC marker, since compared with $CD44^{-}$cells, $CD44^{+}$ cells are more clonogenic and tumorigenic and express high levels of several "stemness genes" encoding the likes of Oct3/4, Bmi1, β-catenin and Smo (Patrawala et al. 2006; Tang et al. 2007).

In brain cancer, clonogenicity (neurosphere-forming) and self-renewal appears exclusive to a minor subpopulation of tumour cells expressing the human neural stem cell markers CD133 and nestin, regardless of the tumour phenotype (Singh et al. 2003). These cells not only have the capacity to differentiate in vitro into cells with neural and glial phenotypes in proportions resembling the original tumour, but their proliferative capacity is also proportional to the aggressiveness of the original tumour. The intracranial injection of as few as 100 $CD133^{+}$ cells from medulloblastomas is able to produce tumours in NOD/SCID mice; these brain tumours can be serially transplanted and are identical to the patient's original tumour (Singh et al. 2004). Islands of $CD133^{+}$ cells are present within tumours, indicating that $CD133^{+}$ cells must be generating both $CD133^{+}$ and $CD133^{-}$ tumour cells; injections of up to 10^{5} CD133-negative cells fail to produce tumours. Studies of neural stem cells have traditionally focused on the subventricular zone, olfactory bulb and hippocampal dentate gyrus, although medulloblastomas might be derived from the cerebellum; a subpopulation of $CD133^{+}$ precursor cells expressing Math1 under the influence of Shh might be the target cells (Lee et al. 2005).

Even in glioblastoma multiforme, a particularly nasty malignant adult human CNS tumour, only a small fraction of $CD133^{+}$ cells is capable of neurosphere generation and multipotential differentiation, with as few as 5,000 of these cells being able to form tumours in nude mice (Galli et al. 2004; Yuan et al. 2004). In this tumour type, $CD133^{+}$ cells are remarkably resistant to irradiation, probably because of an enhanced DNA damage response (Bao et al. 2006).

In common with many tumours, CNS tumours may have an SP fraction likely to be enriched for CSCs. They have been found in the rat C6 glioma cell line (Kondo et al. 2004) and in a great variety of human primary tumours (Hirschmann-Jax et al. 2004, 2005). In two human liver tumour cell lines, a small (<1%) SP fraction exists, and transplantation of as few as 1,000 of these cells is able to generate xenografted tumours in NOD/SCID mice (Chiba et al. 2006). Other studies of HCC cell lines have implied that $CD133^{+}$ cells are enriched for in vitro clonogenicity and in vivo tumour-initiating activity (Suetsugu et al. 2006; Yin et al. 2007).

Most gastrointestinal cell lines also contain a small SP fraction that can self-renew, possibly being the CSCs (Haraguchi et al. 2006). However, other studies point to $CD133^{+}$ cells as being the CSCs of colorectal cancers (CRCs); maybe they are one and the same? $CD133^{+}$ cells comprise about 2.5% of CRC cells (Ricci-Vitiani et al. 2007), whereas CD133 positivity enriches 200-fold for tumour-initiating activity over all tumour cells (O'Brien et al. 2007). Recently, Dalerba et al. (2007) have indicated that the combination of $(epCAM)^{high}CD44^{+}$, possibly combined with $CD166^{+}$, substantially enriches for tumour-initating cells in human CRC.

Table 1 illustrates the phenotypes that have been described for some normal stem cells and their malignant counterparts.

Finally, there is the question of whether, within an individual tumour, are all CSCs the same. The answer is probably not, providing that the evidence from AML that CSCs are heterogeneous in their renewal kinetics is applicable to other tumour types (Hope et al. 2004). The kinetics of renewal of AML cells has been studied by examining their repopulation of NOD/SCID mice, with each initial transplanted cell bearing a unique "signature" from its retroviral insertion site. Some CSCs provide only short-term engraftment, not appearing in mice secondarily transplanted, whereas others seem to renew abundantly,

Table 1 Some proposed markers of normal human stem cells and their malignant counterparts (*GFAP* glial fibrillary acidic protein, *SSEA-1* stage-specific embryonic antigen-1, *ESA* epithelial surface antigen, *CALLA* common acute lymphoblastic leukaemia antigen, *ALDH* aldehyde dehydrogenase, *EpCAM* epithelial cell adhesion molecule, *CML* chronic myeloid leukaemia, *SP* side population); taken from Alison et al. (2006a, b)

Tissue/cancer	Normal tissue stem cells[a]	Cancer stem cells[b]
Brain/medulloblastoma	$CD133^+$nestin$^+$GFAP$^+$	Sub-set of $CD133^+$ pool
	$A2B5^+$	
	ABCG2	$CD133^+$ Math1$^+$
	Musashi1	
	SSEA-1	
Breast/adenocarcinoma	$\alpha6^+$CK19$^+$ESA$^+$MUC1$^-$CALLA$^-$	ESA$^+$CD44$^+$CD24$^{-/low}$
	β-1 integrin$^+$	
	Musashi1	
Haematopoietic/CML	CD34$^+$CD38$^-$	CD34$^+$CD38$^-$
	$CD133^+$	
	ALDH$^+$	CD34$^+$ ALDH$^+$
	SP	
Gastrointestinal/	Musashi1	SP - found at low frequency (0.3–2%) in 15/16 cell lines
Colorectal adenocarcinomaa	Hes-1	$CD133^+$
	Nuclear β-catenin	EpCAM^{high}CD44$^+$CD166$^+$
Prostate/adenocarcinoma	SP/ABCG2	CD44$^+$ $\alpha2\beta1^{high}$ $CD133^+$
	$CD44^+$	
	$CD133^+$	
	CK5/18	
	p63	

[a] Normal stem cells have been much more thoroughly investigated than CSCs and a number of markers have been proposed for each; this is just a sample

[b] Able to propagate the tumour in immunocompromised mice when relatively few cells have been transplanted

being found in primary, secondary and tertiary transplanted recipients of "marked" leukaemic cells. On the other hand, some cells only arise belatedly in secondary or even tertiary transplants, suggesting a much slower rate of renewal.

Molecular regulation of normal stem cells and CSCs: implications for cancer treatment

For tomorrow's oncologist, a variety of druggable targets related to CSCs present themselves and include:

- Wnt signalling
- Hedgehog signalling
- Notch/Delta signalling
- ABC transporters
- Differentiation therapy
- Targeting the stem cell niche
- Small molecule cancer therapeutics

A molecular link between CSCs and normal stem cells seems logical, since neoplastic proliferation appears to be caused by mutations that dysregulate normal self-renewal pathways and that thus affect proliferation (Reya et al. 2003). Proliferation of CSCs is likely to be attributable to a dysregulation of the pathways involved in normal stem cell self-renewal, such as the Wnt-/β-catenin, PTEN, Notch and Hedgehog pathways, and to the products of the *Bmi1* and other Polycomb genes. In leukaemia, mutations frequently seem to tip the proliferative balance of normal HSCs towards an increase in proliferation and reduction in differentiation.

The Wnt/β-catenin pathway, in addition to being pivotal in embryogenesis, is involved in all of the stem-cell-defining characteristics: self-renewal, proliferation and differentiation. The signalling cascade is initiated when extracellular Wnt molecules bind to Frizzleds, their receptors on the cell membrane. This causes an inhibition of the β-catenin destruction complex and consequently an accumulation of β-catenin, which then translocates to the nucleus where it activates the transcription of proliferation- and differentiation-promoting genes by binding to LEF/TCF transcription factors. In vitro cultures of HSCs over-expressing stabilised β-catenin proliferate with a 100-fold increase (Reya et al. 2003) and the addition of purified Wnt3a protein renders in vitro cultures of HSCs six-fold more likely to proliferate and reconstitute the haematopoietic system of irradiated mice, but less likely to differentiate and express differentiation markers (Willert et al. 2003). The essential role of Wnt signalling in vivo is confirmed by the finding that mice lacking Wnt3a or β-catenin are not viable. The importance of Wnts in the proliferation and migration of epithelial stem cells in the normal gut has also been documented by the absence of intestinal stem cells in transgenic mice that lack the Tcf4 transcription factor (Korinek et al. 1998). Moreover, ectopic expression of axin, an inhibitor of Wnt signalling leads to the inhibition of proliferation, increased death of HSCs in vitro and reduced reconstitution in vivo (Reya et al. 2003). In humans, dysregulation of the Wnt signalling cascade has been implicated in cancers of the colon, prostate and ovary. Wnt signalling can be inhibited by bone morphogenetic

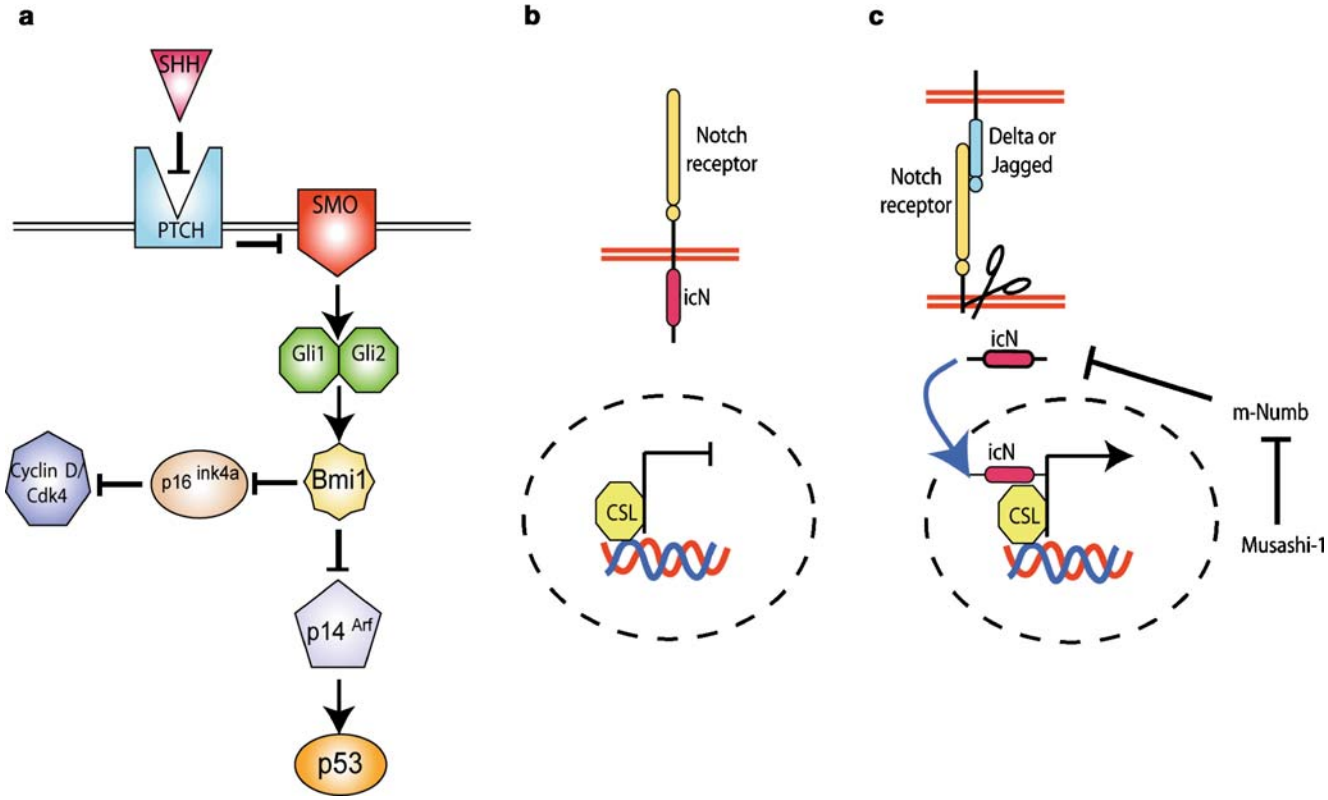

Fig. 4 **a** Representation of Hedgehog signalling, which might help maintain the stem cell state. **b**, **c** Representation of the Notch signaling system, which might maintain stem cell self-renewal. **b** In the absence of Notch ligands, the DNA-binding protein *CSL* acts as a transcriptional repressor. **c** Upon ligand binding, proteolytic cleavage of the intracellular domain of Notch (*icN*) occurs followed by its translocation to the nucleus where it converts CSL to a transcriptional activator. Downstream targets include the repressor *Hes1*, which in turn represses differentiation-inducing genes such as *Math1*. The *m-Numb* protein binds icN, probably targeting it for degradation. The RNA-binding protein *Musashi1* (putative stem cell marker) represses the translation of *m-Numb* RNA, thus potentiating Notch signaling

proteins (BMPs), which are produced by pericryptal mesenchymal cells in the gut (He et al. 2004); disruption of BMP signalling results in an expansion of the stem and progenitor cell compartment leading to a juvenile-polyposis-like state.

In the CNS and haematopoietic system, key regulators of stem cell renewal appear to be members of the Polycomb group protein family of transcriptional repressors (Bmi1, Rae28, Mel-18). Bmi1 targets genes such as $p16^{INK4A}$ and $p14^{ARF}$ preventing stem cell senescence by respectively maintaining cyclinD/Cdk4 signalling and Mdm2 destruction of p53 (Park et al. 2004). Bmi1 is a downstream target of the morphogen Sonic hedgehog (Shh) through the latter's activation of the Gli family of latent transcription factors (Fig. 4a). Shh acts on the receptor complex of Patched (PTCH) and Smoothened (SMO), blocking the restraining influence of PTCH on SMO, resulting in SMO signalling activating the Gli family of transcription factors and so activating target genes such as *Bmi1*. Inhibition of the action of SMO with the anatogonist cyclopamine is a highly effective strategy against a wide range of foregut cancers (Berman et al. 2003). In the skin, mutations in *PTCH* characterise human nevoid BCC (basal cell carcinoma) syndrome (also known as Gorlin's syndrome) and, clearly, Shh signalling in follicular outer root sheath cells leads to BCC, a tumour characterised by a marked lack of features of terminal differentiation (Owens and Watt 2003).

The Notch family of receptors is also critical for stem cell self-renewal, for example in HSCs (Karanu et al. 2005), and in mammary epithelial stem cells (Dontu et al. 2004). Engagement of ligands of the Delta and Jagged families causes cleavage (mediated by the γ-secretase protease complex) of the intracellular portion of Notch and its translocation to the nucleus where it binds to the transcription factor CSL, changing it from a transcriptional repressor to an activator (Fig. 4b, c). The use of γ-secretase inhibitors may have utility in cancers where Notch-signalling is inappropriately activated (van Es and Clevers 2005). Table 2 illustrates some of the abnormalities of the self-renewal machinery of stem cells found in human tumours.

Apart from the renewal pathways, many other potential molecular targets have been associated with CSCs. As many tumours have SP fractions that are almost certainly enriched for CSCs, targeting ABC transporter activity will be an obvious strategy for overcoming chemoresistance and for directly eradicating stem cells (Seigel et al. 2005; Wang

Table 2 Examples of signalling pathways that are involved in normal stem cell renewal and that appear to be dysregulated in human tumours (*FAP* familial adenomatous polyposis, *APC* adenomatous polyposis coli, *CRC* colorectal cancer, *HCC* hepatocellular carcinoma, *JPS* juvenile polyposis syndrome, *BMPR1A* bone morphogenetic protein receptor-1A, *BCC* basal cell carcinoma, *PTCH* Patched, *SCLC* small cell lung cancer, *GIT* gastrointestinal tract, *icN* intracellular Notch)

Disease	Alteration	Consequence
Wnts and related molecules (Reya and Clevers 2005; Zurawel et al. 1998; Moon et al. 2004; Salahshor and Woodgett 2005; Logan and Nusse 2004)		
FAP	*APC* mutation	Activation of β-catenin signalling
Sporadic CRC Endometrial carcinoma Medulloblastoma	β-Catenin mutation	Activation of β-catenin signalling
HCC, CRC, medulloblastoma Adenoid cystic carcinoma	Loss of function mutations in axin	Activation of β-catenin signalling
Lung carcinoma	Over-expression of Dishevelled	Activation of β-catenin signalling
JPS	Mutations in *SMAD-4* and *BMPR1A*	Loss of restraint on Wnt signalling
Hedgehogs (Hh) and related molecules (Gorlin 2004; High and Zedan 2005; Marino 2005; Watkins and Peacock 2004; Kasper et al. 2006; Gil et al. 2005; Valk-Lingbeek et al. 2004)		
Nevoid BCC syndrome (BCC, medulloblastoma, primitive neuroectodermal tumours); sporadic medulloblastoma and others	*PTCH* mutations	Constitutive over-expression of Hh pathway
BCC, SCLC, tumours of cerebellum, skeletal muscle, pancreas, upper GIT, prostate	Over-expression of PTCH ligands or other components of Hh pathway	Constitutive over-expression of Hh pathway
Medulloblastoma, non-SCLC, haematological malignancies	Over-expression of Polycomb group proteins e.g. Bmi1 (inhibiting INK4A and ARF)	Continued cell proliferation and maintenance of the "stem cell state"
Notch receptors and their ligands (Kanemura et al. 2001; Pece et al. 2004; Sjolund et al. 2005; Reedijk et al. 2005)		
Gliomas	Over-expression of Musashi1 represses translation of Notch inhibitor m-Numb	Enhanced Notch signalling
Breast cancer	Enhanced m-Numb degradation	Enhanced Notch signalling
T cell acute lymphoblastic leukaemia	Translocation or point mutation of Notch1 receptor gene; expression of truncated icN independent of Notch ligands	Constitutive Notch signalling
Breast cancer, medulloblastoma, renal cell carcinoma, Hodgkin's disease, pancreatic and prostate cancer	Over-expression of Notch and/or ligands, and other proteins of Notch cascade	Enhanced Notch signalling

et al. 2007). Eliciting the differentiation of CSCs is an attractive option and, of course, retinoic acid has been used successfully for the treatment of certain leukaemias for many years. In human glioblastomas, the inhibition of the BMP/BMP-receptor signalling pathway leads to a reduction in the $CD133^+$ population, reduced proliferation and increased expression of differentiation markers (Piccirillo et al. 2006).

Antibody-based targeting of CSCs exploiting the over-expression of the likes of CD133 and CD44 is also possible, although targeting CD44 could have other effects. Leukaemic stem cells appear to occupy specific niches in trabecular bone (Ninomiya et al. 2007) and, in a murine model of Bcr-Abl^+ CML, blockade of CD44 substantially impedes the homing and engraftment of leukaemic stem cells in the bone (Krause et al. 2006). CSCs in medulloblastoma appear to reside in areas rich in blood vessels, the so-called "vascular niches"; here, targeting endothelial cells cause a dramatic reduction in CSC number (Calabrese et al. 2007). Thus, whereas anti-angiogenic therapy is not new, being used to debulk tumours through disruption of their blood supply, the destruction of the CSC niche adds a new twist to the story.

Small molecule therapeutics that target growth factors, growth factor receptors and their kinases, and more specific tyrosine kinase inhibitors (such as imatinib) that target BCR-ABL-positive CML and c-Kit-positive gastrointestinal stromal tumours are increasingly popular therapeutic

options. Of course, such strategies are not necessarily specific for CSCs. In CML, modelling the response to imatinib suggests that the stem/progenitor cells preferentially survive (Michor et al. 2005); this may be partly attributable to mutations in the hybrid gene (Jiang et al. 2007) but is probably more commonly attributable to the relatively slow cycling nature of the leukaemic stem cells. A recent study suggests that growth factor treatment aimed at pushing the leukaemic stem cells into the cycle may increase the effectiveness of imatinib treatment of CML (Holtz et al. 2007).

Indirect roles for BMDCs in tumorigenesis

BMDCs may indirectly influence tumour behaviour by contributing to the desmoplastic response and to the tumour vasculature (Fig. 5). Endothelial progenitor cells (EPCs) constitute a unique population of peripheral blood mononuclear cells derived from BM and are involved in postnatal angiogenesis during wound healing, limb ischaemia, post-myocardial infarction, atherosclerosis and tumour vascularisation. HSCs and EPCs are derived from a common precursor called an haemangioblast (Khakoo and Finkel 2005). Circulating EPCs are mobilised endogenously in response to tissue ischaemia or exogenously by cytokine therapy to augment neovascularisation. The development of a vascular supply to a tumour is a prerequisite for tumour survival, allowing for the provision of oxygen and nutrients

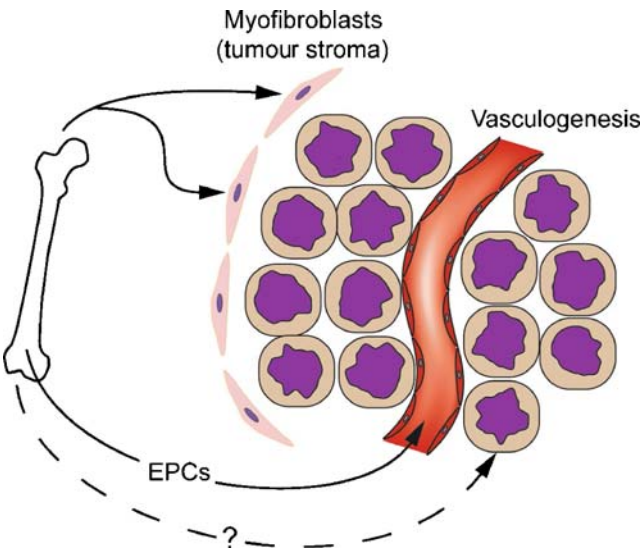

Fig. 5 In the context of tumours, the myofibroblasts and fibroblasts comprising the tumour stroma can be derived from bone marrow, as can many of the endothelial cells of the neovasculature (vasculogenesis). Gastric carcinomas in mice have been found to arise from BMCs engrafted in the gastric epithelium. Whether many human carcinomas have a similar origin is unknown but will be difficult to ascertain (*EPCs* endothelial progenitor cells)

and for the disposal of waste products. New vessel formation is also required for tumour metastasis. Previously, tumour vasculature was thought to develop exclusively via endothelial cell migration and proliferation: angiogenesis. However, the creation of new blood vessels by EPCs is known as vasculogenesis and such a process is a significant event in tumorigenesis.

The reported extent of the BM contribution to tumours is highly variable. In "inhibitor of differentiation" mutant mice in which tumour vasculogenesis is impaired through a failure of EPCs to mobilise in response to tumour-secreted VEGF, transplantation with Rosa 26 BM cells results in the tumour endothelial population being almost 100% $LacZ^+$ (Lyden et al. 2001). On the other hand, in murine mammary tumours, despite long periods (several months to >1 year) after BM transplantation and mammary tumour development, the contribution of BM to the $CD31^+$ endothelial population is never more than 1.9%, with a mean of 1.3% (Dwenger et al. 2004). In two other murine studies, the contribution of BM to tumour vasculature has been shown to be less than 1% or non-existent (Larrivee et al. 2005; Shinde Patil et al. 2005), although the animals were sacrificed within 2 weeks of tumour cell transplantation. A study of murine liver and pancreatic tumours has suggested that only advanced tumours recruit BM cells into the developing vasculature (Hammerling and Ganss 2006).

Little data are available concerning the contribution of BM to human tumour vasculature. However, in the rare clinical setting of tumours developing in female patients who have previously had a male BM transplant, BM has been found to contribute to tumour endothelium, albeit at a low level (average 4.9%; Peters et al. 2005).

BM also contributes to myofibroblast populations in tumour stroma. Direkze et al. (2004, 2006) have shown that the BM can contribute to myofibroblasts and fibroblasts in the tumour stroma in a mouse model of pancreatic insulinoma. Rat-insulin-promoter large-T-antigen (RIPTag) mice develop β−cell tumours of the pancreas. In female RIPTag mice transplanted with male BM, male α-smooth muscle actin (αSMA)-positive cells, i.e. donor-derived myofibroblasts, have been found to comprise almost 25% of the total myofibroblast population (Direkze et al. 2004, 2006). The source of these BM-derived myofibroblasts is likely to have been blood-borne fibrocytes, cells that make up 0.1%–0.5% of non-erythrocytic cells in the peripheral blood (Metz 2003); these cells are $CD34^+$ and express collagen I.

A more surprising role of BM cells in the tumorigenic process has been as emissaries, "preparing the ground" for metastatic colonisation (Steeg 2005). Kaplan et al. (2005) have found that Lewis lung carcinoma cells and B16 melanoma cells cause VEGF receptor-1 ($Flt\text{-}1^+$), $VLA\text{-}4^+$ ($\alpha4\beta1$ integrin) BM cells to set-up premetastatic niches in

the lung; moreover, tumour-specific growth factors upregulate fibronectin (a VLA-4 ligand), providing a "docking site" for the circulating BM cells.

BM transplantation is successfully used in the treatment of haematological malignancy but the contribution of BM to tumour stroma and vasculature provides potentially new portals for the application of anti-cancer therapies: in effect, by using stem cells as Trojan horses. For example, with regard to tumour fibroblasts, Studeny and colleagues have used BM-derived mesenchymal stem cells as vehicles for interferon (IFN) delivery into tumours. They found that mesenchymal stem cells with forced expression of IFN-β inhibit the growth of a variety of xenografted tumours including malignant melanoma, breast cancer and intracranial gliomas (Studeny et al. 2002, 2004; Nakamizo et al. 2005). Concerning the input of BM to tumour vasculature, Davidoff et al. (2001) transduced murine BM with a soluble truncated form of VEGF receptor-2 to mop-up VEGF and then transplanted them into recipient mice with neuroblastomas. The mice with the modified BM transplants had significantly reduced tumour growth indicating the potential of BM as a vehicle for transporting antiangiogenesis molecules directly to the tumour vascular bed. On the other hand, De Palma et al. (2005) have indicated a profound anti-tumour effect through the ablation of BM-derived Tie2 (angiopoietin receptor tyrosine kinase)-expressing mononuclear (TEM) cells, a population of proangiogenic monocytes,. This group have found no evidence that any BM cells integrate into the tumour vasculature but rather that BM-derived Tie-2-expressing cells closely associate with the tumour vasculature and have a significant effect through the paracrine release of the likes of basic fibroblast growth factor. Selective ablation of this specific BM population by expression of thymidine kinase under the control of the transcriptional regulatory elements of the *Tie-2* gene and subsequent ganciclovir treatment (suicide gene therapy) lead to a significant impedance in the growth of various tumours.

Future directions

As it is beyond reasonable doubt that CSCs do exist in most tumours, the next issue to be addressed must be the characterisation of CSCs in order to target them specifically for therapeutic purposes (Fig. 6). The renaissance in stem cell biology for regenerative medicine is likely to improve our understanding of CSCs. We are beginning to unravel the molecular pathways of normal stem cell self-renewal, which are frequently dysregulated in many human cancers. Targeting these pathways of self-renewal in CSCs therefore seems a particularly promising therapeutic strategy. The contribution of the BM to tumour stroma and thus tumour behaviour is a significant factor with obvious therapeutic implications. Moreover, a direct role of BM-derived stem cells in the histogenesis of carcinomas, if found to be a common occurrence, would represent a paradigm shift in carcinogenesis theory. On a cautionary note, a recent paper

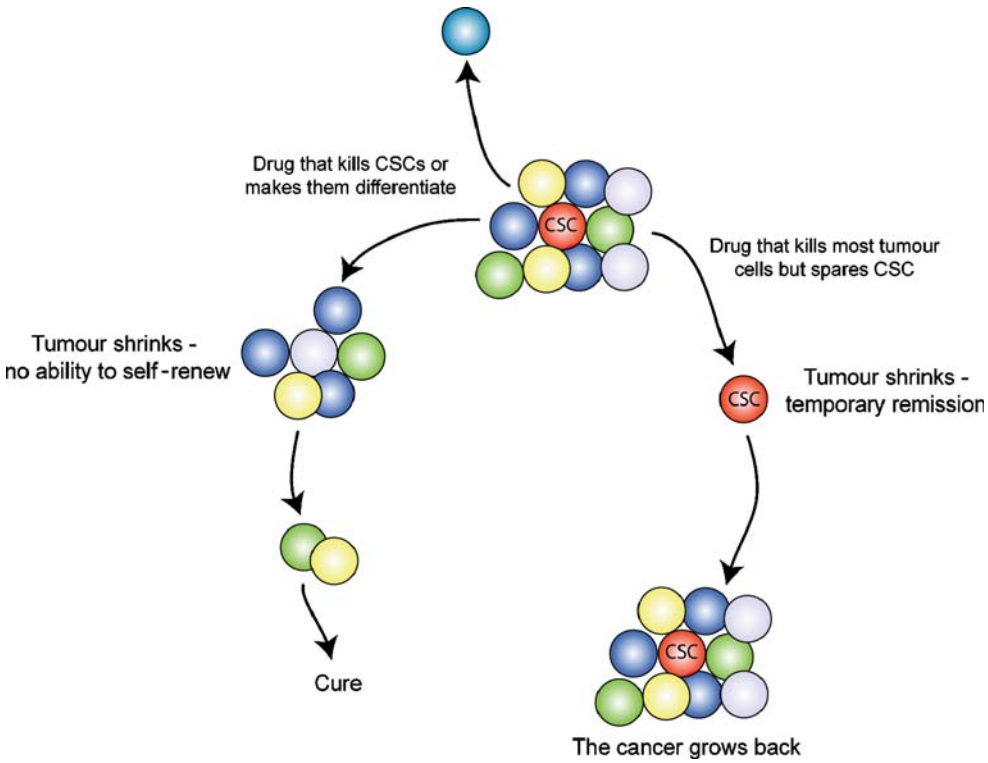

Fig. 6 Successful tumour therapy will involve the direct ablation of CSCs; alternatively, promotion of their differentiation will have the same effect

suggests that BM cells often incorporate into tumours, taking on the phenotype of the tumour cells in a sort-of "developmental mimicry" but not indicating an origin of the cancers from BM cells (Cogle et al. 2007).

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