Cancer stem cells in solid tumours: accumulating evidence and unresolved questions

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Abstract | Solid tumours are an enormous cancer burden and a major therapeutic challenge. The cancer stem cell (CSC) hypothesis provides an attractive cellular mechanism to account for the therapeutic refractoriness and dormant behaviour exhibited by many of these tumours. There is increasing evidence that diverse solid tumours are hierarchically organized and sustained by a distinct subpopulation of CSCs. Direct evidence for the CSC hypothesis has recently emerged from mouse models of epithelial tumorigenesis, although alternative models of heterogeneity also seem to apply. The clinical relevance of CSCs remains a fundamental issue but preliminary findings indicate that specific targeting may be possible.

Solid tumours account for the major cancer burden, and epithelial cancers arising in tissues that include breast, lung, colon, prostate and ovary constitute approximately 80% of all cancers. Tumours are generally characterized clinically at the gross level by histology and by the expression of specific markers. Together with gene expression profiling, this has enabled the definition of distinct tumour subtypes. The cellular origins of most solid tumours are largely unknown, but it has been speculated that different subtypes reflect distinct cells of origin at the time of tumour initiation. In addition to the acquisition of genetic and epigenetic mutations, interactions between tumour cells and their microenvironment (stroma, inflammatory cells and recruited vasculature) have a profound influence on the tumorigenic process. Finally, metastasis and tumour dormancy characterize many solid tumours but the nature of these complex processes remains largely undefined.

In addition to different tumour subtypes, cells within the tumour population itself often exhibit functional heterogeneity, with cells exhibiting distinct proliferative and differentiative capacities (referred to as tumour heterogeneity)[1]. The cellular mechanisms underlying tumour heterogeneity are the subject of intense research in the cancer biology field. In historical and rather extraordinary studies by Southam and Brunschwig[2], evidence was provided for heterogeneity within tumours by autologous transplantation of malignant cells from patients with different carcinomas into subcutaneous tissue. Furthermore, pioneering studies of spontaneous mouse leukaemias and lymphomas revealed that the frequency of tumour-propagating cells ranged from 1% to the majority of cells[3,4]. Functional heterogeneity among cancer cells derived from lung, ovary and brain tumours was also evident in colony-forming assays *in vitro*[5]. At least two models have been put forward to account for heterogeneity and inherent differences in tumour-regenerating capacity: the cancer stem cell (CSC)[6,7] and clonal evolution models[8,9].

This Review summarizes and evaluates the current evidence for the existence of CSCs in solid tumours. We compare the CSCs that have been prospectively isolated from diverse solid tumours, with emphasis on those studies that have used freshly isolated tumour specimens for transplantation. The frequency of CSCs in solid tumours appears to be substantially higher than that in leukaemia but marked variation occurs between tumours of the same type, emphasizing the requirement for more definitive markers and clonality studies. This article also discusses the progress in delineating pathways that regulate CSC function and evidence for a distinct metastatic CSC population. Finally, we review data on the sensitivity of CSC-enriched subsets to antineoplastic agents and the central question of the clinical relevance of the CSC — will CSCs have a major impact on cancer patient management in the future?

Models for tumour propagation

CSCs refer to a subset of tumour cells that has the ability to self-renew and generate the diverse cells that comprise the tumour[6,7,119]. These cells have been termed cancer stem cells to reflect their 'stem-like' properties and ability

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At a glance

- The cancer stem cell (CSC) hypothesis is an attractive model to account for the functional heterogeneity that is commonly observed in solid tumours. It proposes a hierarchical organization of cells within the tumour, in which a subpopulation of stem-like cells is responsible for sustaining tumour growth.
- The first evidence for CSCs came from acute myeloid leukaemia. There is now increasing evidence for CSCs in a variety of solid tumours (both mouse and human), provided through transplantation studies using prospectively isolated tumour cells.
- The frequency of CSCs in solid tumours is highly variable, reflecting biological variation as well as technical issues. Technical issues include the purity of solid tumour cell fractionation, the requirement for more definitive markers and the challenges associated with xenotransplantation. Ultimately it will be necessary to study CSCs and potential heterogeneity within this population at a clonal level through 'cell tagging'.
- Not all solid tumours will follow the CSC model of heterogeneity. Some may conform to the clonal evolution model, in which a dominant population of proliferating cells drives tumorigenesis.
- Metastatic CSCs may exist, with properties distinct from primary CSCs.
- The concept of CSCs has significant clinical implications: CSCs have been shown to be more resistant to chemotherapy and radiotherapy.
- Recent reports, primarily for haematopoietic malignancies, suggest that CSCs can be selectively targeted without ablating normal stem cell function.

to continually sustain tumorigenesis. CSCs share important properties with normal tissue stem cells, including self-renewal (by symmetric and asymmetric division) and differentiation capacity, albeit aberrant. Multilineage differentiation, however, is not a mandatory feature of a CSC. One implication of the CSC model is that cancers are hierarchically arranged with CSCs lying at the apex of the hierarchy[6] (FIG. 1). The first evidence for the existence of CSCs came from acute myeloid leukaemia[6,10], in which a rare subset comprising 0.01–1% of the total population could induce leukaemia when transplanted into immunodeficient mice.

The clonal evolution model postulates that mutant tumour cells with a growth advantage are selected and expanded, with cells in the dominant population having a similar potential for regenerating tumour growth[8] (FIG. 1). The acquisition of genetic events underpins this model but epigenetic differences and microenvironmental changes are also likely to have important roles. The clonal evolution model may, in some cases, involve a stochastic component[7]. Both paradigms of tumour propagation are likely to exist in human cancer but only the CSC model is hierarchical. It is important to note that the two models are not mutually exclusive, as CSCs themselves undergo clonal evolution, as shown for leukaemia stem cells[11]. Thus, a second, more dominant CSC may emerge if a mutation confers more aggressive self-renewal or growth properties (FIG. 1). It is relevant that serial transplantation has been reported to result in the *in vivo* selection of cells that generate more aggressive tumours[12].

CSCs are distinct from the cell of origin. The cell of origin specifically refers to the cell type that receives the first oncogenic hit(s). Moreover, CSCs do not necessarily originate from the transformation of normal stem cells. CSCs may arise from restricted progenitors or more differentiated cells that have acquired self-renewing capacity. One corollary of this model is that there will be mechanistic parallels between the self-renewal programmes of normal stem cells and CSCs (BOX 1). It has been presumed in many cases that the cells in which cancer originates are committed cells that have undergone some degree of differentiation. In haematopoietic malignancies, the MOZ–TIF2 (also known as MYST3–NCOA2) (REF. 13), mixed lineage leukaemia (MLL)–AF9 (REFS 14,15) and MLL–ENL[16] fusion products all seem to confer stem-like properties on committed progenitor cells, leading to the generation of CSCs. For example, there is evidence that MLL–AF9 activates a self-renewal programme in granulocyte–macrophage progenitors, transforming them into leukaemia stem cells[14]. However, in a recent knock-in mouse model of *Mll1–Af9* only HSCs that expressed high levels of the translocation product were efficiently transformed, indicating the importance of gene dosage in determining phenotype[17]. In patients with chronic myeloid leukaemia (CML) blast crisis, a β-catenin mutation also appears to confer self-renewal properties on the granulocyte–macrophage progenitor[18]. In solid tissues, however, definitive cell surface immunophenotypes have not yet been defined for most stem cells and their progeny, making it impossible to determine which cell types the CSCs most closely resemble.

Prospective isolation of CSCs from solid tumours

The most convincing demonstration of CSC identity comes from serial transplantation of cellular populations into animal models, necessitating the development of orthotopic transplantation assays. The CSC-containing population should re-establish the phenotypic heterogeneity evident in the primary tumour and exhibit self-renewing capability on serial passaging. There have been significant technical issues compounding the isolation of CSCs from epithelial and other solid tumours, in part owing to the difficulty in dissociating these cancers. Further, in the case of xenotransplantation, incomplete immunosuppression or species-specific differences in cytokines or growth factors probably present confounding issues. Even in syngeneic models, implantation of tumour cells into a normal niche does not precisely recapitulate the tumour environment itself.

Nevertheless, mounting data over recent years have indicated the existence of CSCs in multiple solid tumours. A number of cell surface markers have proved useful for the isolation of subsets enriched for CSCs, including CD133 (also known as PROM1), CD44, CD24, epithelial cell adhesion molecule (EpCAM, also known as epithelial specific antigen (ESA) and TACSTD1), THY1 and ATP-binding cassette B5 (ABCB5), as well as Hoechst$_{33342}$ exclusion by the side population cells (BOX 2). CSCs have frequently been isolated using markers specific for normal stem cells of the same organ. A summary of the key features of CSC populations prospectively isolated from solid tumours is given in TABLE 1. It is evident that common cell surface markers, in particular CD133 and CD44, have been used to fractionate CSCs in diverse solid tumours. It is not yet known whether these represent surrogate markers or have a role in regulating CSC function. Notably, none of these markers are exclusively expressed by the solid tumour CSC, highlighting the imperative to delineate more specific markers or to use combinatorial markers.

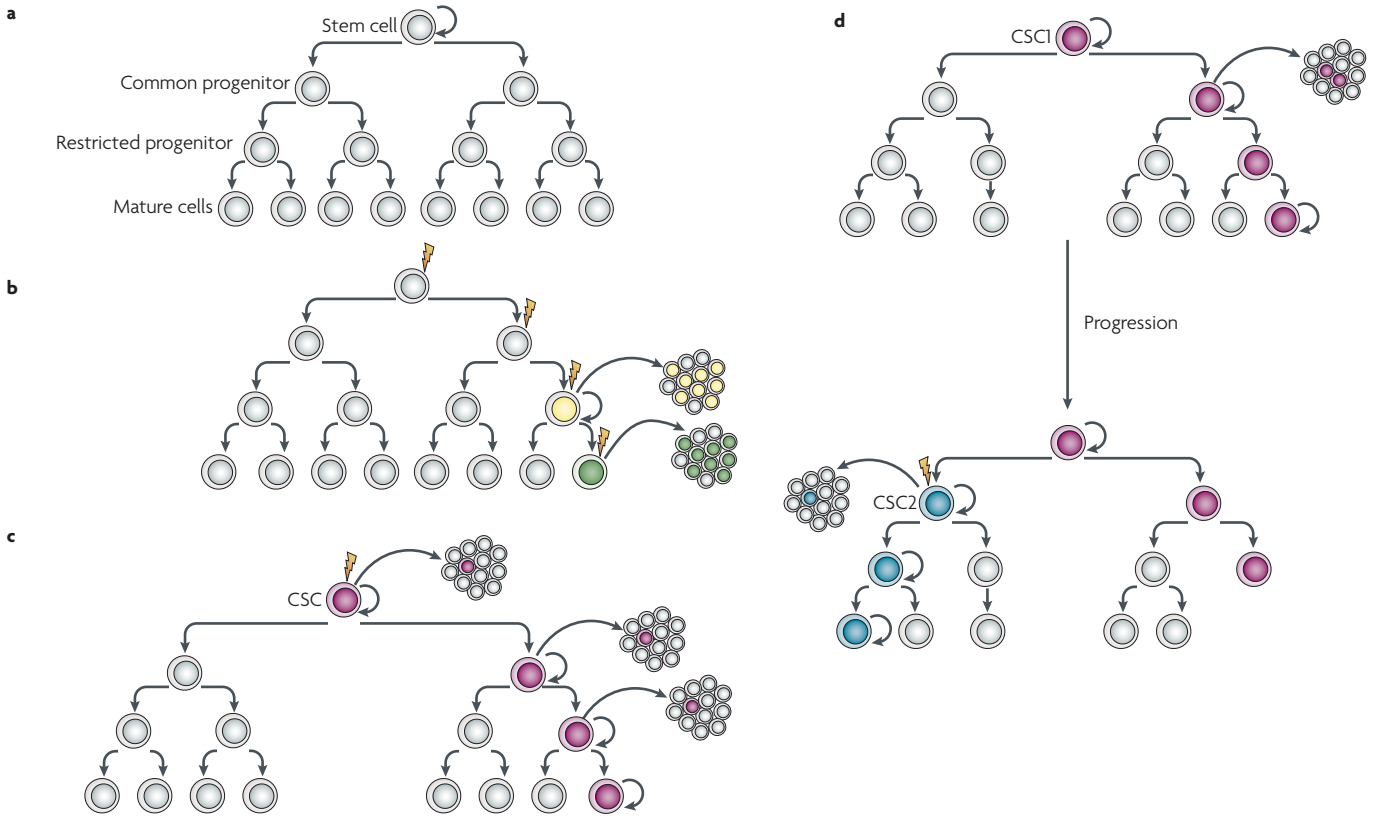

Figure 1 | **Two models for tumour heterogeneity and propagation. a** | A normal cellular hierarchy comprising stem cells (at the apex), which progressively generate common and more restricted progenitor cells, ultimately yielding all the mature cell types that constitute a particular tissue. **b** | In the clonal evolution model all undifferentiated cells have similar tumorigenic capacity. **c** | In the cancer stem cell (CSC) model, only the CSC can generate a tumour, based on its self-renewal properties and enormous proliferative potential. **d** | Both models of tumour maintenance may underlie tumorigenesis. Initially, tumour growth will be driven by a specific CSC (CSC1). With tumour progression, another distinct CSC (CSC2) may arise due to clonal evolution of CSC1. This may result from the acquisition of an additional mutation or epigenetic modification. This more aggressive CSC2 becomes dominant and drives tumour formation.

The first solid malignancy from which CSCs were identified and isolated was breast cancer. Al-Hajj *et al.*[19] described a $CD44^+CD24^{-/low}$ cell population that was significantly enriched for tumour-initiating capacity. Subsequently, CD133 was found to mark CSCs in different types of brain tumours including glioblastoma multiforme, paediatric medulloblastoma and ependymomas[20–25]. In addition, CD133 has been instrumental in identifying CSCs in colorectal[26,27] and pancreatic[28] carcinomas. CD133 itself appears to be a marker of normal neural stem cells in both human[29] and mouse[30] and possibly pancreatic stem cells[31].

Some overlap, albeit limited, is evident between the cell surface phenotypes of CSCs described thus far by different groups for the same tumour type. In pancreatic cancer, for example, although overlap was found between the $CD133^+$ and $CD44^+CD24^+$ populations, it was highly variable: 10–40% of $CD44^+CD24^+$ cells in primary tumours were positive for CD133 expression[28]. On the other hand, the $EpCAM^{hi}CD44^+$ CSC subset in colorectal cancer[32] exhibits little overlap with CD133, and generally constitutes a minor proportion of the $CD133^+$ population. In breast carcinomas, high aldehyde dehydrogenase 1 (ALDH1, also known as ALDH1A1) activity was recently shown to identify the tumorigenic fraction[33]. However, the $ALDH1^+$ population shows a surprisingly small overlap with the previously described $CD44^+CD24^{-/low}$ phenotype (0.1–1.2%)[19]. Nonetheless, the combination of these cell surface markers with Aldefluor yielded a highly enriched population, with as few as 20 cells required to generate a tumour. One source of variation in marker expression may be attributable to culturing of cells before cell sorting rather than the use of fresh material.

Heterogeneity within CSC populations

Phenotypic heterogeneity within CSC subpopulations is likely to exist. In gliomas, CD133 expression does not always appear to mark the CSC[22]. Indeed, glioblastomas could be propagated from $CD133^-$ cells, although these cells were cultured as adherent spheres and cell lines before transplantation and long-term self-renewal was not measured. In this study, $CD133^-$ cells were similarly tumorigenic to $CD133^+$ cells in nude mice, although $CD133^-$ tumour cells appeared to have a distinct molecular profile[22].

Box 1 | **Signalling pathways in cancer stem cells**

The same pathways appear to orchestrate self-renewal and proliferation in diverse stem cell compartments and to cause neoplasia when deregulated. The normal self-renewal programs include the Wnt, Notch, Hedgehog and BMI1 pathways. Alternatively, tumour suppressors that inhibit tumour proliferation or regulate cellular responses to DNA damage, such as p53, PTEN, and INK4A and ARF (both encoded by *CDKN2A*), may also block stem cell self-renewal[104,105]. The precise pathways activated in cancer stem cells (CSCs) in solid tumours have not been elucidated, but PTEN and INK4A have been implicated in gliomas, and sonic hedgehog (SHH) in basal cell carcinomas and gliomas (reviewed in REF. 106). BMI1 overexpression and mutations in SHH pathways may be involved in the genesis of medulloblastoma, and SHH signalling can increase BMI1 expression in primitive neural stem or progenitor cells[107]. Active repression of differentiation genes by polycomb proteins such as BMI1 may be required for self-renewal of CSCs. Do CSCs have a definitive signature of self-renewal genes that can transform a differentiated cancer cell into a CSC?

In intestinal stem cells, the canonical Wnt pathway regulates self-renewal and maintains the stem cell niche in conjunction with bone morphogenetic protein (BMP) and Notch signalling[108]. Mutations that activate Wnt signalling lead to hyperproliferation of crypt progenitors and generation of benign polyps[108]. Wnt signalling also regulates the migration and proliferation of stem cells and progenitors through pathways involving ephrin family adhesion proteins[109]. On the other hand, transforming growth factor β and BMPs function as negative regulators of stem cells, preventing their activation and proliferation. BMP4 appears to be expressed in the intestinal and bulge epidermal stem cell niches and targeted deletion of the BMPR1A receptor results in expansion of stem cells in the intestine and hair follicle with consequent tumour development[110–112]. Thus, cross-talk between positive and negative regulatory signals can govern stem cell function.

In skin tumours, conditional deletion of β-catenin from either chemical- or Ras-induced tumours resulted in complete regression of the tumours[49], establishing a crucial role for the Wnt–β-catenin pathway in maintenance of the $CD34^+$ CSC population. There appears to be a direct link between WNT1 signalling and the DNA damage response in primary human breast epithelial cells[113]. In *MMTV–Wnt1* mice, constitutive β-catenin signalling results in mammary tumours and increased progenitor cell numbers, and further appears to render these progenitor cells radioresistant[92]. An expansion of the mammary stem cell pool was also noted during the pre-neoplastic phase in *MMTV–Wnt1* transgenic mice, implicating these stem cells as the putative target of transformation[114]. Notably, activation of the WNT1–β-catenin pathway can confer self-renewing properties on luminal progenitor cells during oncogenesis[47]. Wnt signalling has a parallel role in haematopoietic malignancies, in which it was recently found to be essential for the renewal of chronic myeloid leukaemia stem cells *in vivo*[115].

The CSC phenotype may not necessarily be uniform between cancer subtypes or even tumours of the same subtype. In one breast cancer patient[19] with a comedo (generally more aggressive) type of adenocarcinoma, the tumorigenic cell population was noted to be $CD44^+CD24^+EpCAM^+$. In this tumour, there may have been a distinct CSC or an alternative model of tumorigenesis could apply. In addition, cell lines derived from *Brca1*-deficient mouse mammary tumours were recently shown to harbour heterogeneous cancer cell populations. Either $CD44^+CD24^-$ or $CD133^+$ cells, dependent on the *Brca1*-deficient tumour, could elicit tumours in immunocompromised mice when injected in low cell numbers (50–100) (REF. 34). Significantly, there was no overlap between the $CD44^+CD24^-$ and $CD133^+$ populations, suggesting that heterogeneity exists within the putative CSC populations of *Brca1* tumours. It will be important to validate these findings on potential CSC heterogeneity using freshly sorted cells.

Tumour xenograft
Owing to the limited amount of tumour material it is necessary to establish xenografts. This involves limited passaging of the tumour, preferably in an orthotopic location, in immunocompromised mice such as NOD-SCID strains. The validity of using xenografts has been documented for many different tumour types. The engraftment rate can be variable, dependent on the tumour type.

Cancer stem cell frequency

The frequency of CSCs appears to be highly variable between tumours of the same type but is substantially higher than that for leukaemia stem cells[6]. For example, the size of the $ABCB5^+$ fraction appears to vary between 1.6 and 20% of total tumour cells among melanomas[35], and the $CD133^+$ CSC population in colorectal carcinomas ranges between 1.8–24.5% (REF. 26). Recent mathematical analyses have further indicated that CSCs in advanced tumours may not occur as a small fraction[36]. More refined markers combined with extensive *in vivo* limiting dilution analysis are required to more accurately determine the frequency of CSCs within solid tumours and to prove enrichment[37]. This may eventually allow correlation between CSC frequency, tumour grade and clinical outcome. A recent study provides evidence that CD133 expression in gliomas predicts poor patient survival and the proportion of $CD133^+$ cells appears to be an independent risk factor for tumour relapse[38]. It is plausible that the clinically aggressive basal subtype of breast tumour may also contain a higher proportion of CSCs but this has not yet been evaluated. Given that the basal subtype shares the hallmark features of mammary stem cells (triple-negative for oestrogen receptor, progesterone receptor and ERBB2), it is tempting to speculate that a CSC arising from transformation of a normal stem cell yields a more aggressive cancer than one derived from a more committed progenitor cell. In highly metastatic tumours for which melanoma serves as a paradigm, the CSC population may have distinct properties that are more reminiscent of a metastatic or migratory CSC (see below).

Putative CSCs in prostate and lung cancer

Hoechst-excluding side population and $CD44^+$ cells sorted from xenografted prostate tumours have been reported to be enriched for tumour-initiating cells[39,40], but serial transplantation studies are yet to confirm whether they represent *bona fide* CSCs. Other studies have identified putative human prostate CSCs with a $CD44^+$α2 integrin$^+$β1 integrin$^{hi}CD133^+$ phenotype based on extensive proliferative capacity *in vitro*[41]. In small-cell and non-small-cell lung tumours[42], CD133-expressing tumour cells (0.32–22% of the total tumour population) were recently identified in four different tumour subtypes. These cells grew indefinitely as spheres and were tumorigenic[42]. Furthermore, high numbers of $CD133^+EpCAM^+$ cells (10^4) isolated from fresh lung tumour specimens were capable of generating tumour xenografts upon subcutaneous injection, but their self-renewal capacity was not clear.

CSCs in mouse models

CSCs do not necessarily constitute a minor component of the tumour. Evidence accumulating from mouse models has indicated that the frequency of CSCs can vary dramatically. Although haematopoietic malignancies are not the focus of this article, such mouse models warrant some discussion in the context of CSC frequencies. CSCs may be relatively infrequent in some mouse models of tumorigenesis. In murine leukaemia models

originating from the MOZ–TIF2 translocation[13], a blast crisis model of CML[43] or *Pten*-deficient mice[44], rare cells have leukaemia-forming capacity. However, recent studies by Kelly *et al.* have shown that >10% of cells in three mouse models of leukaemia or lymphoma have tumour-regenerating capacity, suggesting that these are maintained by a dominant cell population[45]. Indeed, the recipients of 3 out of 8 single-cell transfers from an *Eμ–Myc* tumour developed lymphoma. Furthermore, at least 25% of granulocyte–myeloid progenitors or myeloid-lineage cells harbouring the *Mll–Af9* oncogene could initiate leukaemogenesis in recipient mice[14,15]. In all of these models, no functionally distinct subpopulation has been identified to date. These tumours may be sustained by a high frequency of CSC-like cells or, alternatively, they may conform to the clonal evolution model of tumorigenesis. Indeed, if every cell in these tumours proves to be a tumour-propagating cell, then neither model of heterogeneity applies.

In the context of mammary tumours, CSC subsets have been described in two different models of mammary tumorigenesis using distinct marker combinations. The use of syngeneic models excludes any bias associated with xenotransplantation systems. In *MMTV–Wnt1* mammary tumours, a $THY1^+CD24^+$ cancer cell population (1–4% of tumour cells) was found to be highly enriched for tumorigenic activity relative to the non-$THY1^+CD24^+$ population[46]. One in every 200 cells from this basal population generated tumours phenotypically similar to the original tumour upon injection near the upper mammary fat pads, and these could be serially passaged in mice. These studies further suggested that the Wnt–β-catenin signalling pathway has an important role in governing the self-renewal of cancer cells in these tumours. Using a different set of markers (β1 integrin, CD24 and CD61 (β3 integrin)), we have also identified a CSC population in *MMTV–Wnt1* mammary tumours that confers 20-fold enrichment in tumour-initiating capacity[47]. In a model of the breast tumorigenesis that mimics breast cancer that develops in Li–Fraumeni patients, the *Trp53*-null mammary tumour model, a subpopulation of cells at the tip of the $\beta1\ integrin^{hi}CD24^+$ fraction was shown to be enriched for CSCs[48]. However, CSCs may not characterize all mouse models of mammary tumorigenesis. In the well-established *MMTV–Erbb2* strain, no CSC subset could be identified using multiple different cell surface markers. These luminal tumours exhibit substantial homogeneity and a frequency of 1 in 100 tumorigenic cells in the total epithelial population, suggesting a clonal evolution or stochastic model of tumorigenesis[47].

Striking evidence for the existence of CSCs in mouse models has come from the study of cutaneous tumours. In mouse skin, bulge stem cells specifically express CD34 and have been demonstrated to maintain follicular homeostasis. Remarkably, $CD34^+$ cells sorted from cutaneous tumours arising from DMBA–TPA-mediated carcinogenesis were shown to be 100-fold more potent in initiating tumours than unsorted tumour cells: 1,000 $CD34^+$ cells induced tumours in syngeneic transplants,

Box 2 | **Markers of cancer stem cells in solid tumours from patients**

ABCG5
Member of the ATP binding cassette family, involved in transport of sterol and other lipids. ABCG2 (also known as breast cancer resistance protein) is a multi-drug transporter (see Hoechst SP below). ABCG5 confers doxorubicin resistance.

ALDH1
The ubiquitous aldehyde dehydrogenase (ALDH) family of enzymes catalyse the oxidation of aliphatic and aromatic aldehydes to carboxylic acids. ALDH1 has a role in the conversion of retinol to retinoic acid, which is important for proliferation, differentiation and survival.

CD24 (HSA)
A heavily glycosylated glycosylphosphatidylinositol-anchored adhesion molecule, which has a co-stimulatory role in B and T cells. The only known ligand for P-selectin. Although CD24 is not a specific marker of cancer stem cells, low levels can characterize breast tumour-initiating cells.

CD44 (PGP1)
An adhesion molecule with multiple isoforms that has pleiotropic roles in signalling, migration and homing. The standard form CD44H exhibits a high affinity for hyaluronate; CD44V confers metastatic properties.

CD90 (THY1)
A glycosylphosphatidylinositol-anchored membrane glycoprotein involved in signal transduction that has a potential role in stem cell differentiation. It may mediate the adhesion of thymocytes to the thymic stroma.

CD133 (prominin 1)
Five-transmembrane domain glycoprotein with a potential role in the organization of plasma membrane topology. Expressed on $CD34^+$ stem and progenitor cells in fetal liver, endothelial precursors, fetal neural stem cells and developing epithelium, CD133 has been detected by its glycosylated epitope in the majority of studies. Thus, AC133 may be a more reliable cancer stem cell marker[116].

EpCAM (epithelial cell adhesion molecule; ESA, TROP1)
Homophillic Ca^{2+}-independent cell adhesion molecule expressed on the basolateral surfaces of most epithelial cells.

Hoechst SP
Side population (SP) phenotype due to the $Hoechst_{33342}$ efflux pump present on the plasma membrane in diverse cell types. Activity conferred by the ABC transporter ABCG2. Inhibited by verapamil.

Table 1 | **Prospective isolation of human cancer stem cells from freshly dissociated solid tumours**

Tumour type	CSC marker	Tumour cells expressing CSC marker, %	Minimal number of cells expressing CSC markers for tumour formation	Injected in Matrigel	Transplantation site	Strain	Refs
Breast	$CD44^+/CD24^{-/low}$	11–35	200	+	Mammary fat pad	NOD-SCID	19
Breast	$CD44^+/CD24^-$	ND	2×10^3	–	Mammary fat pad	NOD-SCID	77
Breast	$ALDH1^+$	3–10	500	+*	Mammary fat pad	NOD-SCID	33
Brain	$CD133^+$ (GBM) $CD133^+$ (MB)	19–29 6–21	100 100	– –	Brain Brain	NOD-SCID NOD-SCID	20 20
Brain	$CD133^+$	2–3	500	–	Brain	nu/nu	24
Colon	$CD133^+$	1.8–25	200	+	Kidney capsule	NOD-SCID	26
Colon	$CD133^+$	0.7–6	3×10^3	–	Subcutaneous	SCID	27
Colon	$EpCAM^{hi}/CD44^+$	0.03–38	200	+	Subcutaneous	NOD-SCID	32
Head and neck	$CD44^+$	0.1–42	5×10^3	+	Subcutaneous	$Rag2/\gamma^{-/-}$DKO, NOD-SCID	117
Pancreas	$CD44^+/CD24^+/ESA^+$	0.2–0.8	100	+	Pancreas	NOD-SCID	65
Pancreas	$CD133^+$	1–3	500	–	Pancreas	NMRI-nu/nu	28
Lung	$CD133^+$	0.32–22	10^4	–	Subcutaneous	SCID	42
Liver	$CD90^+$	0.03–6	5×10^3	–	Liver	SCID/Beige	53
Melanoma	$ABCB5^+$	1.6–20	10^6	–	Subcutaneous	NOD-SCID	35
Mesenchymal	Side population (Hoechst dye)	0.07–10	100	–	Subcutaneous	NOD-SCID	118

*Also injected with fibroblasts. ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; EpCAM, epithelial cell adhesion molecule; ESA, epithelial specific antigen; GBM, glioblastoma multiforme; MB, medulloblastoma, ND, not determined; NOD-SCID, non-obese diabetic-severe combined immunodeficient; $Rag2/\gamma^{-/-}$DKO, Rag 2 common cytokine receptor γ-chain double knockout.

whereas 2.5×10^4 $CD34^-$ cells could not[49]. This orthotopic transplantation system also included primary keratinocytes and dermal fibroblasts to allow whole-skin reconstitution. The tumours exhibited the same hierarchical organization as the parent tumours and the $CD34^+$ subset had self-renewing capacity, fulfilling the definition of a true CSC.

The high frequency of transplantable tumour-propagating cells (>10%) in certain mouse models of haematopoietic malignancy may reflect the more homogeneous nature of these lymphomas and leukaemias. These generally arise with much shorter tumour latency than solid tumours, presumably contributing to less heterogeneity within their cancer cell population. Nevertheless, the absolute frequency of CSCs is likely to vary markedly between different solid tumours and, in some cases, a different model of tumorigenesis may apply. Although the cell hierarchies are likely to be similar between mouse and human, mouse models may not always be representative of spontaneously occurring human malignancies, such as those models where the oncogenic event has occurred in a cell type that differs from that in human disease. The choice of genetically engineered mouse model and the cell type in which the promoter or enhancer driving the transgene is active will undoubtedly have major influence on the frequency of transplantable cells.

Are there metastatic cancer stem cells?

Metastasis is the predominant cause of lethality in cancer patients. However, not every cell in a tumour has the ability to metastasize to other organs. Metastatic potential depends on multiple factors that determine overall tumour cell growth, survival, angiogenesis and invasion. For epithelial malignancies, the epithelial–mesenchymal transition (EMT) is considered to be a crucial event in the metastatic process, which involves disruption of epithelial cell homeostasis and the acquisition of a migratory mesenchymal phenotype[50]. In many epithelial tumours, an EMT or loss of differentiation is frequently evident at the invading edge of the tumour and is likely to mediate cellular detachment and eventual metastasis[50]. The EMT appears to be controlled by canonical pathways such as the Wnt and transforming growth factor β pathways, both of which can be aberrantly activated during neoplasia. A recent report suggests that there may be a direct link between the EMT and acquisition of stem cell properties[51]. Cells undergoing an EMT could conceivably be the precursors to metastatic cancer cells, perhaps even metastatic CSCs. CSCs may also have a role in the creation of a particular niche for metastasis. It is notable that primary tumour cells can indeed generate a pre-metastatic niche by recruiting haematopoietic progenitor cells to tumour-specific niches[52].

Can metastases directly arise from CSCs? Recent data have supported the concept of a metastatic CSC. Hermann *et al.* defined a distinct subset of $CD133^+CXCR4^+$ cells (CXCR4 is the receptor for the chemokine CXCL12 (also known as SDF1)) that localized to the invasive edge of pancreatic carcinomas and exhibited significantly stronger migratory activity *in vitro* than $CD133^+CXCR4^-$ cells despite both subsets

showing similar tumour development[28]. Significantly, only CD133$^+$CXCR4$^+$ cells demonstrated *in vivo* metastatic activity to the liver. Although the study was performed using a pancreatic cancer cell line, the concept that there are functionally distinct subsets of cells that impart either tumour-propagating or metastatic activity seems compelling. Moreover, pharmacological depletion of CD133$^+$CXCR4$^+$ cells by inhibition of the CXCR4 receptor profoundly reduced the metastatic potential of pancreatic tumours without altering their tumorigenic potential. Primary pancreatic tumours comprised a higher fraction of CXCR4$^+$ cells that displayed increased migratory activity *in vitro* and, furthermore, correlated with metastatic disease in these patients. These findings have important therapeutic implications, as the design of drugs that target the metastatic CSC would be envisaged to have a profound effect on patient survival. In hepatocellular cancer, CSCs that might directly contribute to metastasis (circulating THY1$^+$ CSC-like cells) have been detected in patients with liver cancer and could generate tumours in immunocompromised mice[53].

In breast cancer patients, CD44$^+$CD24$^{-/low}$ cells are readily detectable in metastatic pleural effusions[19], although there is often a substantial percentage of non-tumour cells present in malignant pleural fluid[54] and this aspect has yet to be addressed in prospective studies. Furthermore, a 186-gene invasiveness signature generated from CD44$^+$CD24$^{-/low}$ cells indicates the propensity of a tumour to metastasize[55]. By contrast, an increased number of CD24$^+$ cells has been reported in distant metastases in breast cancer patients despite the CD44$^+$ basal cell signature predicting decreased patient survival and a higher risk of distant metastases in lymph node-negative patients with invasive breast cancer[56]. Although there are few data on cellular mechanisms mediating the metastasis of breast cancer cells, it is possible that CD44$^+$CD24$^{-/low}$ breast cancer cells initially metastasize and then change their phenotype following limited differentiation at their new site. In general, the CD44$^+$ and CD24$^+$ cell populations within the same tumour were found to be genetically identical, although one pleural effusion presented an exception. In metastatic colon cancer, CD133 does not seem to specifically mark the CSC subset, as both CD133$^+$ and CD133$^-$ cells generated tumours and CD133$^-$ cells formed more aggressive tumours[57]. Collectively, these findings could reflect the generation of a distinct metastatic CSC within the tumour or the evolution of a second CSC with a different immunophenotype from the first CSC (FIG. 2).

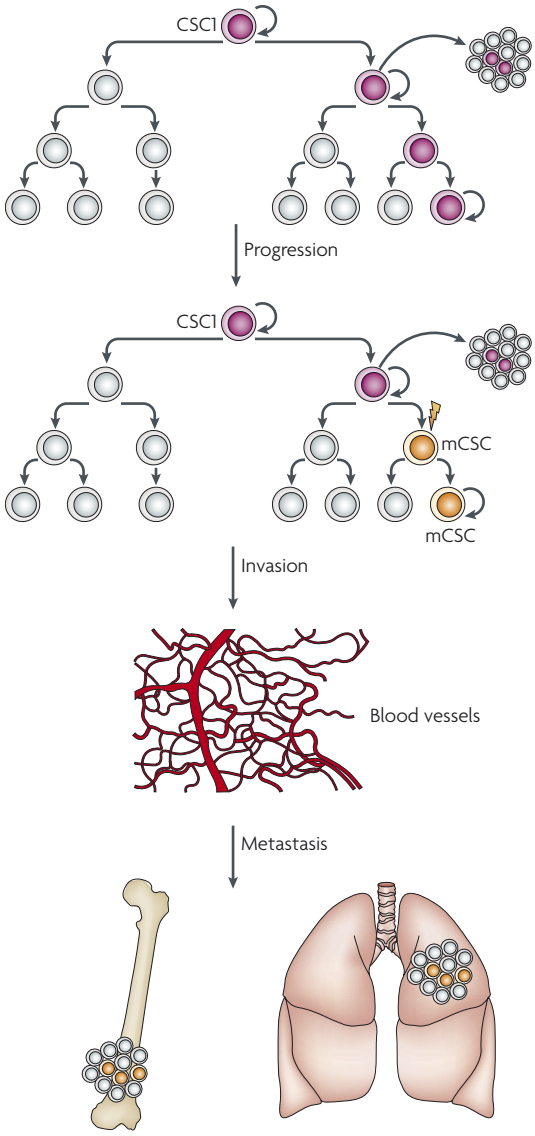

Figure 2 | **Evolution of a metastatic cancer stem cell (CSC).** As a tumour progresses, genetic and epigenetic mechanisms may result in the emergence of a self-renewing metastatic CSC (mCSC) that expresses different cell surface markers from the CSC that is driving tumorigenesis. This mCSC, through a series of invasive processes that characterize metastasis, enters the blood stream and seeds a secondary tumour in a distinct organ.

Issues associated with transplantation

Difficulties imposed by the nature of the xenograft model. The efficiency of xenotransplantation, in the majority of cases, is considerably lower than that for syngeneic transplants. This is partly due to species-specific differences in the affinity (or recognition) of cytokine and growth factor receptors for their cognate ligands. A recent study by Kelly *et al.*[45] has raised the possibility that rare CSCs may be inadvertently selected during xenotransplantation, whereas the majority of cells are incapable of surviving in their foreign environment owing to the lack of appropriate supporting factors. However, there are some murine growth factors that are important stimuli for stem and progenitor cells in multiple organs and can interact well with their equivalent human receptors, such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2)[58,59]. Although there are limitations associated with xenografting, it seems likely that cells within many tumour types may receive sufficient signals from their host milieu. Sufficient time must be allowed for tumour development to occur following xenotransplantation and this is an important parameter when interpreting data.

The importance of both species- and tissue-specific influences is highlighted by the studies of Kuperwasser *et al.*[60]. This work involved 'humanization' of the mouse mammary fat pad through the pre-injection of human breast fibroblasts into cleared mouse mammary fat pads to recapitulate the predominance of these cells in human breast tissue relative to the mouse mammary gland. Although outgrowths were generated in the humanized fat pads, the repopulating frequency by normal breast stem cells remains relatively low and highlights the challenge of recapitulating the human microenvironment in the mouse[33,60]. Similar arguments also apply to the tumour cell niche. Mixing experiments using same-patient cancer-associated fibroblasts with breast tumour epithelial cells may reconstitute the niche more effectively. The use of $CD133^+$ endothelial cell precursors or mesenchymal stem cells may offer further improvement. Other tumour cells are also likely to cooperate with CSCs in tumour progression. Moreover, the systemic environment is clearly important but this is not necessarily apparent at first. For example, oestrogen has been found to profoundly affect the growth of oestrogen receptor-negative breast cancers[61]. Circulating oestrogens led to recruitment of bone marrow-derived stromal cells and promoted the growth of tumours in virgin mice. However, recapitulation of the human systemic environment in a mouse model poses a significant challenge.

Residual immune effector cells. Another factor that can significantly influence the efficiency of human cell engraftment is the presence of residual immune effector cells in recipient mice. For example, non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice show better engraftment than SCID mice owing to a polymorphism in the NOD *Sirpa* allele. This polymorphism confers support for human haematopoiesis in NOD-SCID mice by mediating enhanced binding to the human CD47 ligand on human haematopoietic stem cells[62], and indicates an essential role for mouse macrophages as mediators of engraftment of these human stem cells. NOD-SCID mice, however, retain some natural killer cell activity. This variable has now been eliminated through the generation of NOD-SCID–IL2Rγ^{null} (interleukin 2 receptor γ) mice, which lack mature lymphocytes and natural killer cells and are long-lived compared with other immunodeficient strains[63]. In these mice, human peripheral blood stem cells engraft at high frequency[63]. Although engraftment is improved by implantation into immunocompromised mice, it is also clear that immune cells have a role in the progression of many tumours. Ultimately, it will be imperative to use human bone marrow to reconstitute the haematopoietic and immune repertoire in mice to improve the efficacy of xenotransplantation.

The site of transplantation influences tumour-initiating frequency. Context is an important factor in determining the tumorigenic frequency and striking differences have been observed when tumour cells were transplanted into different microenvironments. Glioblastoma-derived cells exhibit take efficiencies of 50% when implanted subcutaneously and 100% when orthotopically implanted into the cranium[64]. Conversely, substantially increased numbers of tumour cells were required for tumorigenesis when injected into the pancreas as opposed to a subcutaneous site, which is not the normal niche for pancreatic cancer cells[65]. This finding suggests that an altered vascular environment can influence the activity of tumour cells, but it is nevertheless important to use an orthotopic transplantation assay to recapitulate the tumour environment as closely as possible. For the colon CSC, an orthotopic transplantation assay has not yet been described, but a marked difference was noted in the tumour-initiating frequency dependent on the site of injection. Under the kidney capsule, the take rate approaches 100%, whereas it is only about 30% if the tumour cells are injected subcutaneously (J. Dick, personal communication). This difference may reflect the highly vascular sub-renal environment. The site of transplantation could also account for the different numbers of cells from the colon CSC-enriched population required to induce a tumour in separate studies. Ricci-Vitiani *et al.*[27] reported that at least 3,000 cells were required to induce a tumour following subcutaneous injection, and O'Brien *et al.*[26] found that 100–200 cells implanted under the renal capsule were sufficient.

Matrigel has been commonly used in xenotransplantation assays, as evident in TABLE 1. As a reconstituted basement membrane, it has primarily been used by epithelial biologists to study invasion by tumour cells, and an altered extracellular matrix can indeed promote tumour development[66]. Matrigel was found to dramatically facilitate tumorigenesis of human breast[67] and squamous cell carcinoma cells[68] in mice and even supported tumour development by cell lines that did not grow under standard conditions[68]. Human teratoma formation in the mouse was also markedly influenced by the presence of Matrigel, such that tumorigenic efficiency following subcutaneous injection was increased from 25% to 100% (REF. 69). It seems that the use of Matrigel for all tumour types is arguably not always physiological, as it may provide tumour cells with additional proliferative or survival signals that they may not encounter in their usual microenvironment. Despite the potentially more permissive environments provided by some implantation sites or Matrigel, it is relevant that only the CSC and not the negative cell fraction from primary tumours induced the formation of tumours in recipient mice (TABLE 1).

Non-adherent sphere assays for CSCs

Although serial orthotopic transplantation studies are the gold standard for both normal cells and CSCs, they require substantial time (FIG. 3). Non-adherent sphere assays are increasingly being used to evaluate stem cell activity in normal tissue as well as putative CSCs. However, in the majority of these serum-free 'sphere' cultures, there is little definitive information as to which cells are being propagated. The defining characteristics of these different spheres and their relationship with normal stem cells have been unclear, causing over-interpretation of results in many cases. This is largely

Non-adherent sphere
Both normal and cancerous cells from numerous organs can be expanded as non-adherent sphere-like cellular aggregates in serum-free media containing EGF and FGF2.

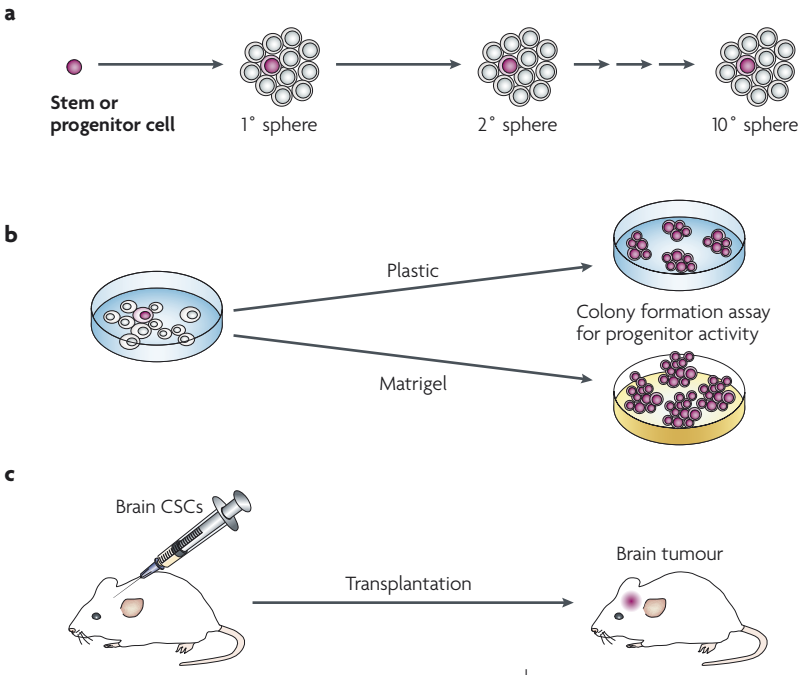

Figure 3 | ***Ex vivo* and *in vivo* assays for tumour cells. a** | The non-adherent sphere assay predicts that a cancer stem cell (CSC) can be serially passaged for many cycles and that it generates a tumour sphere resembling the primary sphere in each case. **b** | Tumour cells may be passaged directly on plastic or embedded in Matrigel, a substitute for the basement membrane. Each colony-forming assay represents a read-out for progenitor cell activity. Stem cells and progenitors cannot be distinguished in these assays. **c** | The gold-standard for evaluating the presence of CSCs is orthotopic transplantation of sorted human subpopulations into an immunocompromised mouse.

because extensive self-renewal (a cardinal feature of stem cells and CSCs) has been difficult to define in the context of a sphere assay[70,71].

The neurosphere has been widely used to study neurogenesis and represents the best-studied sphere assay, both in terms of clonality and multipotent differentiation. There is clear evidence that neurospheres can be derived from stem as well as progenitor cells and that neurosphere frequency approximates progenitor cell activity more closely than stem cell activity[70,71]. Extensive self-renewal demonstrated over more than five passages provides an indicator of neural stem cell activity but it is not useful for quantifying stem cell frequency. Moreover, some assays have been performed with large numbers of cells and therefore not under clonal conditions. An alternative colony-forming cell assay in a collagen matrix may provide a better read-out of neural stem cells based on differential proliferative potential[70]. Although neural stem cells have largely been identified on the basis of their ability to self-renew and form multilineage colonies *in vitro*, the same cells may exhibit significantly different properties *in vivo*[72].

In the context of sphere assays for tumour cells, a number of groups have found that glioblastomas efficiently form tumour spheres in a clonogenic manner[20,22,24,73]. $CD133^+$ cells in brain tumours have a greater tendency to form neurospheres than $CD133^-$ cells and, moreover, the most aggressive clinical samples of medulloblastoma demonstrated the highest secondary sphere-forming capacity[20,24,120]. Using this sphere assay, Hedgehog–Gli signalling has been implicated in regulating the self-renewal and tumorigenesis of $CD133^+$ CSCs in gliomas and could be partially inhibited by cyclopamine, an inhibitor of the Hedgehog pathway[74]. Further, a chemical genetics screen for inhibitors of neurosphere proliferation has revealed small molecules that potently inhibit cultures enriched for CSCs[75]. Unexpectedly, temozolomide was recently shown to preferentially eliminate CSCs in human glioblastoma cells cultured *in vitro* as spheres and to substantially reduce tumorigenicity *in vivo*, suggesting that it could be effective in eradicating CSCs in conjunction with other therapies[76]. Remarkably, $CD133^+$ CSCs isolated from colon cancers could be maintained *in vitro* as undifferentiated tumour spheres for more than 1 year and these retained tumour-initiating capacity[27]. Although it remains to be determined whether non-adherent spheres selectively enrich for CSCs, this assay may provide a useful and predictive model of the therapeutic response of CSC-containing tumours to a specific drug or compound before testing *in vivo*.

Repeated passaging of cell lines for many generations frequently leads to change of characteristics and the acquisition of genetic aberrations, resulting in the selection of atypical clones. The distinction between tumorigenic and non-tumorigenic cell populations can therefore become blurred. Moreover, cell surface markers on tumorigenic cells in established cell lines may differ from CSCs in primary tumours. Thus, observations made in cancer cell lines must be extended to primary tumours in order to validate their significance.

A malignant variant of the human breast cancer cell line SK-BR3 was generated upon serial passaging of cells in NOD-SCID mice treated with the chemotherapeutic agent epirubicin[77], using the mammosphere assay developed by Wicha and colleagues[78]. The drug-resistant cell line was 100-fold more tumorigenic than the parental SK-BR3 line, exhibited a >100-fold increase in the proportion of $CD44^+CD24^-Lin^-$ cells and could be continually grown as mammospheres *in vitro*. Serial transplantation of primary cancer xenografts in mice, either treated or untreated with chemotherapy, will be required to exclude the possibility that a dominant clone has been selected from this established cell line. It is noteworthy that for some breast cancer cell lines sphere-selected cells were no more tumorigenic *in vivo* than cells grown on plastic, and that cells from malignant pleural effusions (from breast cancer patients) did not give rise to tumours after more than 10 months following fat-pad implantation, despite generating spheres in culture (B. Vonderhaar, personal communication).

Therapeutic implications of CSCs

From a clinical perspective, the CSC concept has significant implications, as these cells need to be eradicated in order to provide long-term disease-free survival. Quiescent CSCs are thought to be more resistant to chemotherapy and targeted therapy. A recent study[79] supports the concept that certain CSCs

may enter a quiescent state, given that the majority of human leukaemia stem cells in xenotransplanted mice were found in the G0 phase of the cell cycle and were resistant to chemotherapy. Furthermore, recent targeting of the PML tumour suppressor eradicated the quiescent leukaemia-initiating cells in CML[80]. In some cases, quiescent tumour stem cells may correspond to dormant cancer cells. Inactivation of the MYC oncogene in a hepatocellular carcinoma model revealed that these tumours can exist in a state of dormancy, possibly identifying the dormant cells as liver CSCs[81]. Most CSCs, however, appear to evade cytotoxic therapies or irradiation through active mechanisms. In other cases, clinical evidence indicates that the emergence of drug-resistant clones is through the acquisition of mutations and clonal evolution, such as the *BCR–ABL* mutations that arise in imatinib-resistant CML.

Although the true clinical relevance of the CSC is yet to be revealed, there are tantalizing reports that the CSC can be selectively targeted without ablating normal stem cells. There are several examples of sensitizers within haematopoietic malignancies. Jordan and colleagues identified the naturally occurring small molecule parthenolide as an agent that selectively targets human leukaemia stem cells and not normal stem or progenitor cells[82]. Rapamycin, which targets mTOR (also known as FRAP1), led to eradication of leukaemia-initiating cells arising due to *Pten* deletion in mice and further restored normal HSC function, which was impaired through disruption of *Pten*[44]. CD44 may also be a therapeutic target for CSCs that express this molecule in solid cancers, as normal haematopoietic stem cells do not appear to rely on this adhesion molecule for their function to the same extent as leukaemia stem cells[83,84]. Indeed, altering CD44 function led to a marked delay in the progression of leukaemia in mouse models and provides a paradigm for targeting CSC niches. Nevertheless, it will be paramount to carefully establish whether such monoclonal antibodies also attack normal stem cells in the cancer patient.

Overall, stem cell maintenance pathways may provide suitable targets to sensitize CSCs to therapy, but only if normal somatic stem cells are unaffected. The stem cell niche itself may confer protection to normal cells against a CSC-targeted therapy but there is little definitive information on this at present. In other cases, the genetic programmes governing self-renewal may be differentially active in normal and malignant stem cells. Of relevance here, β-catenin signalling was shown to be essential for sustaining skin CSCs but not normal follicular bulge epidermal stem cells. This mechanistic distinction could lead to specific targeting of CSCs in squamous cell carcinoma[49].

In one strategy directed at eradicating CSCs in glioblastomas, Piccirillo *et al.*[25] showed that bone morphogenetic proteins (BMPs) could induce differentiation of $CD133^+$ cells predominantly to astrocyte-like cells, markedly attenuating their tumour-forming ability. BMP exposure depleted the $CD133^+$ cell fraction *in vitro*, leading to a more differentiated phenotype. Transplantation of $CD133^+$ tumour cells previously cultured with BMP or implanted with beads soaked in BMP (either co-transplantation or post-orthotopic injection) reduced tumour growth and increased the survival of xenografted animals. Significantly though, some CSCs escaped differentiation by BMP, resulting in the death of some mice at 3 months after transplantation. A continuous dose of BMPs therefore seems to be required to ensure differentiation of the entire CSC pool. Deregulation of BMP signalling in gliomas through epigenetic silencing may lead to inhibition of differentiation of tumour-initiating cells, conveying pro-proliferative signals[85].

There is increasing interest in the possibility of exploiting the putative CSC niche for drug targeting. CSCs may dictate expansion of the normal niche as they proliferate (FIG. 4). This may eventually lead to an altered niche as the cells become independent of normal regulatory signals and produce extrinsic factors that deregulate niche-forming cells. Even though there is substantial evidence for an instructive role of the tumour microenvironment, the existence and architecture of the CSC niche remain elusive. Nevertheless, aberrant stem cell niches may result in disease, as exemplified by an altered haematopoietic stem cell niche leading to the development of myeloproliferative disease[86]. Moreover, glioblastoma and medulloblastoma CSCs appear to be maintained by signals from an aberrant vascular niche that mimics the normal stem cell niche. Parallel findings by Calabrese *et al.*[87] and Bao *et al.*[21] showed that freshly isolated $CD133^+$ CSC-enriched cells but not $CD133^-$ glioblastoma cells formed highly vascular tumours in the brains of immunocompromised mice. Treatment of $CD133^+$ cells with bevacizumab, a vascular endothelial growth factor-neutralizing monoclonal antibody, markedly inhibited their ability to initiate tumours *in vivo* and depleted both blood vessels and self-renewing $CD133^+$ cells from tumour xenografts. These studies suggest that glioblastoma CSCs have potent angiogenic activity. Thus, anti-angiogenic therapy in conjunction with cytotoxic chemotherapy may prove effective in targeting CSCs in glioblastomas[88].

Studies on whether CSCs in solid tumours are more chemoresistant or radioresistant than the bulk population are most advanced for brain cancer. Bao *et al.*[24] demonstrated that $CD133^+$ cells in fresh glioblastoma specimens or glioma xenografts irradiated *in vivo* were more resistant to ionizing irradiation than $CD133^-$ cells. Thus, an expansion in the $CD133^+$ subset was found following irradiation both *in vitro* and *in vivo*. Notably, they observed that $CD133^+$ cells preferentially activated the DNA damage checkpoint response more effectively than $CD133^-$ cells in human glioma xenografts and primary glioblastoma specimens. Therefore, this CSC population appears to have evolved a more efficient DNA damage repair system than the bulk of the tumour, conferring resistance to radiation treatment. Most recently, inhibition of the PI3K pathway was found to sensitize putative CSCs in the perivascular niche of medulloblastoma to radiation-induced apoptosis[89]. Brain CSCs may also be augmented by hypoxia. Hypoxia has been shown to have

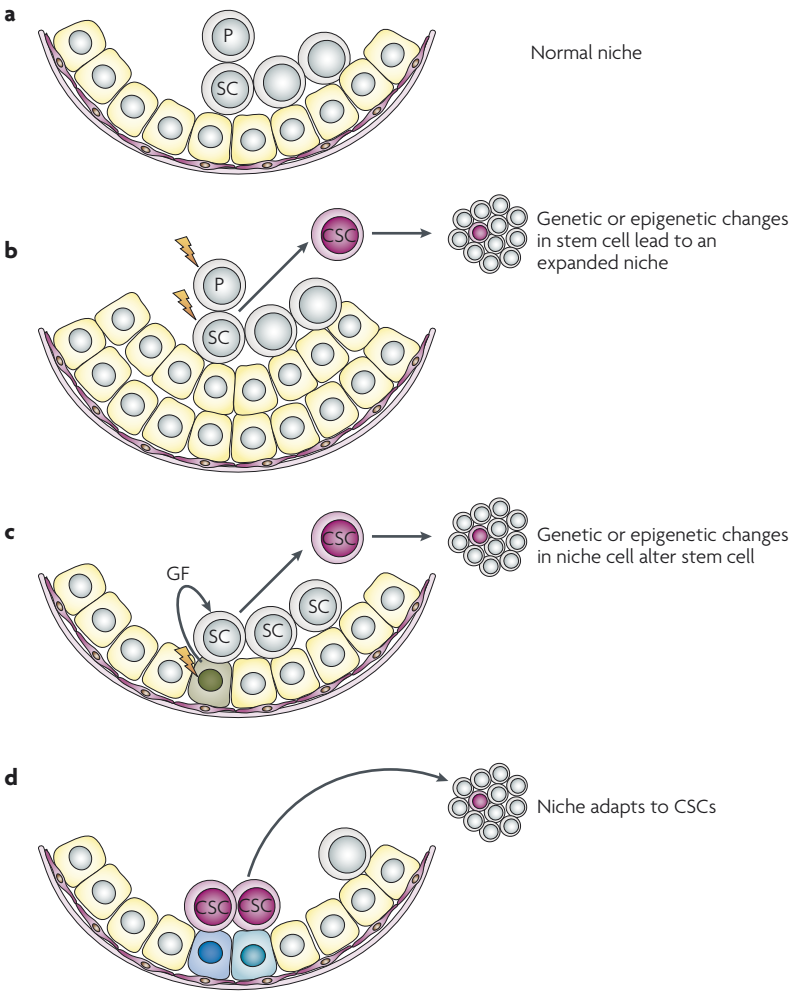

Figure 4 | **Reciprocal interactions between the cancer stem cell (CSC) and its microenvironment or niche. a** | Normal niche containing a stem cell (SC), progenitor cell (P) and supporting cells. **b** | A series of genetic and epigenetic changes occur in a stem cell (or committed progenitor cell), leading to the generation of a CSC. This leads to expansion of cells within the niche. **c** | Genetic and epigenetic changes occurring in a cell within the niche (green) results in the inappropriate production of a growth factor (GF), for example, which eventually leads to the generation of a CSC. **d** | The niche adapts to the presence of CSCs, with cells changing their properties (blue) and/or the recruitment of cells that would not normally be present. In (**b**), (**c**) and (**d**), the architecture of the niche has been altered through the genesis of a CSC.

an important role in defining sensitivity to radiation[90], perhaps through promoting CSC maintenance. Radioresistance has also been implicated in putative breast CSC populations[91,92].

Recent attempts to enrich for breast tumour-forming cells have taken advantage of the apparent therapeutic resistance of CSCs[93]. In chemotherapy-treated patients, ~74% of tumour cells had the $CD44^{+}CD24^{-/low}$ phenotype compared with 9% of cells from untreated patients[77]. Enrichment of these putative CSCs was also evident on analysis of paired specimens from seven patients taken before chemotherapy and then following neoadjuvant chemotherapy. In a larger study, clinical evidence for a subpopulation of chemotherapy-resistant breast CSCs was obtained, lending support to the CSC hypothesis[94]. In the context of mouse tumours, CSCs in *Brca1*- and p53-mediated mouse mammary tumours may contribute to cisplatin resistance[95]. For pancreatic carcinoma, treatment of mice carrying tumour xenografts with gemcitabine, a drug frequently used to treat patients with pancreatic cancer, also revealed that the $CD133^{+}$ CSC population is more resistant[28]. Recent serial transplantation studies have revealed that CSCs in colorectal cancers are substantially enriched following chemotherapy and have increased ALDH1 activity that mediates resistance to cyclophosphamide[96]. Using a different mechanism, some colon CSCs appear to resist cell death through the production of IL4 and antagonism of signalling along the IL4–IL4Rα axis sensitizes these cells to chemotherapeutic drugs[97].

The mechanisms underlying drug resistance are poorly understood but various stem cells often express higher levels of drug-resistance proteins such as ATP-binding cassette half-transporter proteins (ABCG2 and ABCG5) and multidrug resistance protein 1 (MDR1) transporters, and augmented levels of these in CSCs may contribute to the refractoriness of metastatic cancer to chemotherapy[98]. Interestingly, expression of the drug transporter and chemoresistance mediator ABCB5 (REF. 35) correlated with clinical melanoma progression, and specific targeting of the $ABCB5^{+}$ subset with a monoclonal antibody significantly inhibited tumour growth[35].

Conclusions

Although there is substantial evidence for the existence of CSCs in both mouse and human carcinomas, many unresolved issues are apparent. CSC fractions in solid tumours remain highly impure populations and the reported frequencies for the same tumour types have varied enormously between different groups, reflecting impurity as well as technical differences. Nonetheless, the CSC frequency is anticipated to differ between individual tumours of the same subtype and to be potentially higher in more aggressive tumours. In any event, more definitive markers are required, as many antigens including CD133 are widely expressed outside of the putative CSC population. It is curious that CD133 marks so many different types of CSCs, as well as normal stem cells, perhaps implying a fundamental role in sustaining the stem cell phenotype. Recent findings using a mouse knock-in model have indicated that CD133 is expressed widely in the colon[57] but questions remain as to whether *CD133* promoter activity correlates with CD133 surface expression and whether expression of the glycosylated form (AC133 epitope) is restricted to CSCs.

There is relatively poor overlap between the different markers reported for CSCs within a given tumour type, such as those in breast and pancreatic tumours. The lack of concordance most probably reflects impure populations. Additional markers may come from gene expression analyses, proteomics or the high-throughput generation of monoclonal antibodies against cell surface antigens. Even though none of the markers defined so far is unique to the CSC, this task may prove impossible for certain tumours. Nevertheless, a combination of refined markers should greatly improve purity, as so

elegantly exemplified for the haematopoietic stem cell. Differences between normal stem cells and CSCs may reveal novel antigenic and molecular targets for therapy. There are indeed phenotypical differences reported between leukaemia stem cells and haematopoietic stem cells, such as THY1 and IL3Rα expression[99,100].

It will be important to evaluate the stability of the CSC immunophenotype over time. In addition, one has to be cognisant that proteolytic enzymes may potentially destroy or modulate the expression of cell surface markers. Recent findings of Shmelkov *et al.* highlight the importance of careful preparation of single cell suspensions[57]. Relevantly, CSC markers often mimic those expressed on the normal cell counterpart, suggesting that proteolytic digestion of solid tumours does not necessarily alter surface immunophenotype. If some markers prove to be transiently expressed or subject to stochastic activation, then relying on prospective isolation of CSCs will not be a valid approach. Intriguingly, clonal heterogeneity (based on cell surface markers) in a haematopoietic progenitor cell line could be re-established from multiple subclones[101], although specific subclones had distinct but fluctuating transcriptomes. If applicable to certain solid tumours, then these would be viewed as following the clonal evolution paradigm of heterogeneity.

Ultimately, it is essential to demonstrate that solid tumours can be formed from a single transplanted CSC, analogous to the lentiviral tagging of acute myeloid leukaemia cells[102] or the single cell transplants carried out with teratocarcinoma cells[103]. Clonal assays are also necessary to determine whether CSCs are heterogeneous and exhibit difference degrees of 'stemness'[102]. Detailed genetic analyses of tumour cell populations (preferably at the single-cell level) in patients at different stages of disease, before and after treatment, and during relapse or therapeutic resistance will provide further validation of the hierarchical model and distinguish those tumours that follow the clonal evolution rather than the CSC paradigm.

An integrated, multifaceted approach with the development of suitable *ex vivo* and *in vivo* models should lead to further characterization of the CSC. High-resolution imaging technology together with stromal markers will improve our understanding of the cellular niche for different CSCs and will facilitate refinement of the transplantation models to more accurately recapitulate the niche of tumorigenic cells. From a clinical perspective, it will be equally important to decipher mechanisms of chemoresistance and radioresistance that operate in CSCs. The generation of mouse models that develop heterogeneous tumours that closely mimic human malignancies and early generation xenograft tumours from patients will provide powerful tools for evaluating the therapeutic sensitivity of CSCs. There is an additional need for tracking residual CSCs following treatment in order to monitor the efficacy of potential anti-CSC-targeted therapies. With advanced *in vivo* imaging technology, it might be possible to visualize tumour cells and their progression. During tumorigenesis, characteristics of the CSC may change and a distinct CSC could eventually emerge, thus presenting a 'moving target'. In practice, combinatorial treatments involving both cytotoxic and targeted therapies will probably be required to ablate all cancer cells, particularly if there is a 'moving target' or if both models of heterogeneity apply.

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DATABASES

Entrez Gene:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Brca1 | *CDKN2A* | *Pten*

National Cancer Institute: http://www.cancer.gov/
breast cancer | colon cancer | ependymomas | glioblastoma multiforme | glioma | lung cancer | medulloblastoma | melanoma | ovarian cancer | pancreatic carcinoma | prostate cancer | teratoma

National Cancer Institute Drug Dictionary:
http://www.cancer.gov/drugdictionary/
bevacizumab | cisplatin | cyclophosphamide | doxorubicin | epirubicin | gemcitabine | rapamycin | temozolomide | verapamil

UniProtKB: http://www.uniprot.org
α2 integrin | β1 integrin | β-catenin | ABCB5 | ABCG2 | ABCG5 | AF9 | ALDH1A1 | BMI1 | BMP4 | BMPR1A | CD24 | CD34 | CD44 | CD47 | CXCR4 | EGF | ENL | ERBB2 | FGF2 | FRAP1 | IL2Rγ | IL3Rα | IL4 | IL4Rα | MDR1 | MLL | MYC | MYST3 | NCOA2 | p53 | PML | PROM1 | SDF1 | SHH | TACSTD1 | THY1 | WNT1

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