OPINION

Unravelling cancer stem cell potential

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Abstract | The maintenance and repair of many adult tissues are ensured by stem cells (SCs), which reside at the top of the cellular hierarchy of these tissues. Functional assays, such as *in vitro* clonogenic assays, transplantation and *in vivo* lineage tracing, have been used to assess the renewing and differentiation potential of normal SCs. Similar strategies have suggested that solid tumours may also be hierarchically organized and contain cancer SCs (CSCs) that sustain tumour growth and relapse after therapy. In this Opinion article, we discuss the different parallels that can be drawn between adult SCs and CSCs in solid tumours.

Tissue-specific stem cells (SCs) are essential for maintaining tissue homeostasis and repair. SCs have been characterized in many different tissues, such as the haematopoietic lineages, digestive tract, muscle, brain and skin. These tissue-specific SCs are functionally characterized on the basis of two essential properties: their long-term self-renewal capacity and their ability to give rise to one or more differentiated cell lineages that ensure the specific functions of these tissues[1]. Before the development of functional assays to assess SC potential, morphological and proliferation analyses suggested that not all cells are equivalent within a given tissue: some cells seem to be more differentiated than others, and not all cells actively proliferate at the same time[2]. Based on these observations, it has been hypothesized that the differentiated cells may originate from proliferating undifferentiated cells.

SCs have mostly been studied on the basis of functional assays such as *in vitro* clonogenic assays and transplantation and lineage-tracing experiments (FIG. 1). The multilineage differentiation potential of tissue-specific SCs was first suggested by the demonstration that some bone marrow cells are able to differentiate into different haematopoietic lineages following their transplantation into irradiated mice[3]. Later, it was shown that epithelial cells, such as keratinocytes, could be cultured on feeder cells and expanded *in vitro*, stimulated to differentiate, transplanted and engrafted long term as a functional tissue[4]. This suggested that adult SCs may be cultured *in vitro* for prolonged periods while retaining their differentiation potential[5]. More recently, *in vivo* lineage tracing has been developed to assess the fate of SCs more directly and within their natural environment, this has demonstrated the crucial role of SCs during development, as well as during tissue homeostasis and repair[6].

For a long time, it has been recognized that not all tumour cells are identical and that in some cancers, such as teratocarcinoma, a proportion of tumour cells are more differentiated than others, which led to the suggestion that the undifferentiated tumour cells are tumour SCs[7,8]. Two models are usually proposed to explain tumour growth and heterogeneity. In the first model, all tumour cells are equipotent and a proportion of tumour cells stochastically proliferate to fuel tumour growth while other tumour cells differentiate. In the second model, tumours are hierarchically organized like normal tissues, only certain cells contribute to long-term tumour growth and progenitors have limited growth potential (FIG. 2). Owing to the analogy to tissue-specific SCs, which are responsible for the maintenance of adult tissues, the tumour cells that contribute to long-term growth have been referred to as cancer SCs (CSCs) and have been proposed to be responsible for the maintenance and the growth of tumours. Importantly, clonal evolution, in which tumour cells progressively accumulate mutations, some of which confer increased fitness and survival advantage and allow the mutated tumour cells to outcompete other tumour cells, can occur in both models[9].

Recently, accumulating evidence has shown that some cancer cells share certain biological and molecular properties with tissue-specific SCs. In this Opinion article, we discuss how different functional assays have revealed that solid tumours are heterogeneous in their proliferative potential and that only a subset of tumour cells presents long-term renewal potential, supporting a proliferation hierarchy among cancer cells. We also discuss how mouse models allowed the characterization of some of the mechanisms responsible for the acquisition and maintenance of tumour SC potential and their implications for cancer therapy.

Tissue and tumour heterogeneity

The first functional experiments to produce results suggesting that epithelial tissues are heterogeneous and composed of cells with different proliferative potential were carried out in the epidermis. Culture of epidermal cells at clonal density on a fibroblast feeder layer demonstrated that keratinocytes give rise to three types of colony. The holoclones present the greatest proliferative capacity, are composed of cells with undifferentiated morphology that can be passaged over long periods, and are thought to contain epidermal SCs. The paraclones have a more restricted proliferative potential, cannot be passaged over long periods, undergo terminal differentiation and are thought to be transit-amplifying progenitors. The meroclones seem to be a transitional stage between the two other types of colonies[10]. By assessing the clonogenic potential of different parts of the skin epidermis, it has been shown that the permanent portion of the hair follicle, called the bulge, is enriched for these holoclones[11]. Interestingly, although bulge SCs present the greatest proliferative potential *in vitro*, these cells are the most quiescent *in vivo*[12]. Transplantation of bulge cells isolated by microdissection or flow cytometry demonstrated that bulge SCs are able to differentiate into the different epidermal lineages[13–16], supporting the idea that bulge cells are multipotent SCs.

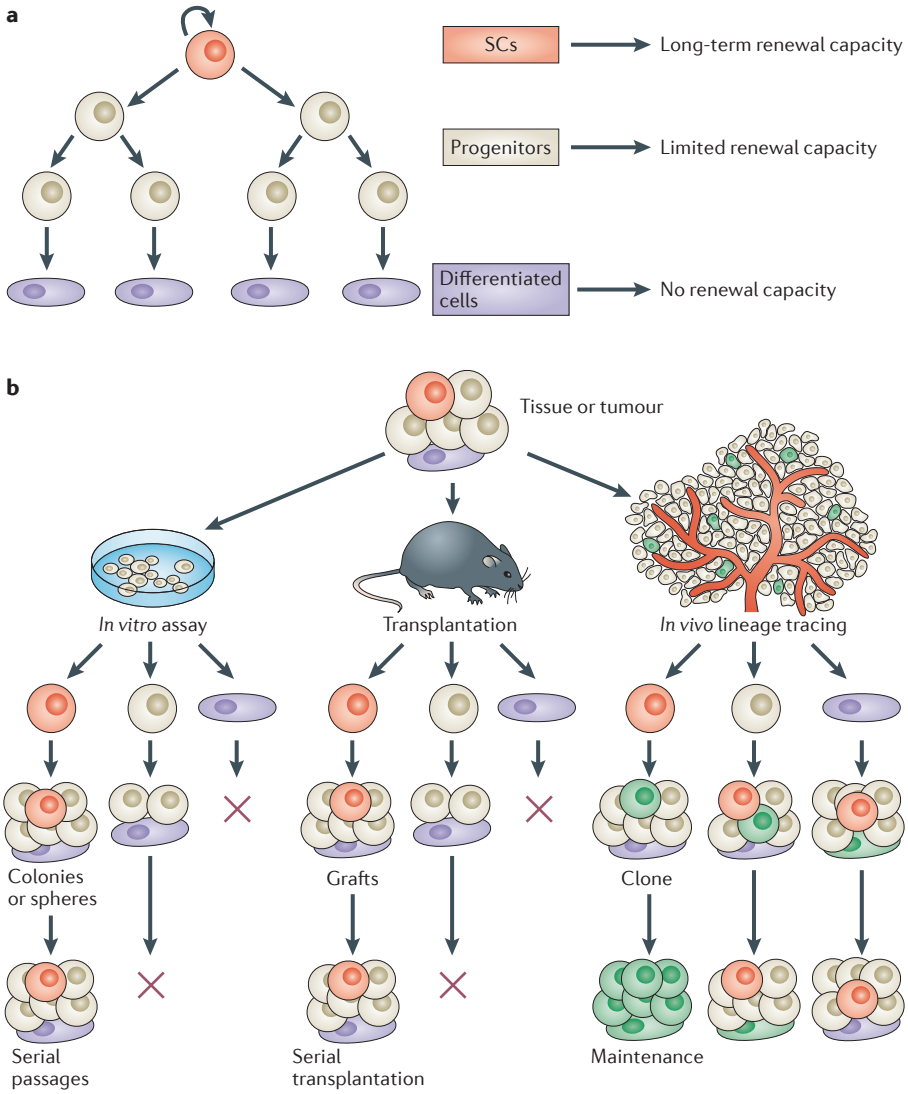

Figure 1 | **Hierarchy in normal tissues and tumours.** **a** | A cellular hierarchy in normal tissues or tumours implies that not all cells are equivalent and that only stem cells (SCs) present long-term self-renewal and differentiation potential. SCs give rise to new SCs and to more committed progenitors, which have a restricted renewal potential and which ultimately give rise to terminally differentiated cells. **b** | Functional assays, such as *in vitro* clonogenic assays, transplantation and lineage-tracing experiments (marked cells are shown in green), can be used to assess the renewal and differentiation potential of SCs, committed progenitors and differentiated cells. Owing to their long-term renewal potential, only SCs or cancer SCs (CSCs) would be able to be serially passaged *in vitro*, to be serially transplanted *in vivo* and to give rise to big clones in lineage-tracing experiments. The committed progenitors give rise to colonies or spheres *in vitro* only transiently, to secondary tumours on transplantation that cannot be serially transplanted, and to small and transient clones in lineage-tracing experiments. Differentiated cells should fail to form colonies or spheres *in vitro*, as well as grafts and clones of multiple cells *in vivo*.

Different *in vitro* clonogenic assays (sphere-forming, organoid culture and co-culture assays) have been developed to address the question of tissue heterogeneity and to study the proliferation, self-renewal and differentiation of different cell populations at the single-cell level. The sphere-forming assay has been used extensively in the field of neuronal SCs to characterize cells that are able to form free-floating spheres, called neurospheres, from adult brain tissue[17]. This assay has been used with many other tissues, including breast, cornea, pancreas, prostate and trachea tissue[18]. Although sphere-forming assays can assess clonogenicity, long-term renewal capacities and multilineage differentiation, it is important to note that not only SCs but also their transit-amplifying progeny are able to form spheres and that, by contrast, quiescent SCs cannot form spheres[18,19]. Therefore, although the sphere-forming assay is a good complementary approach to assess SC proliferation and differentiation, it does not allow for the accurate quantification of SC frequency *in vivo*. More recently, culturing cells in a three-dimensional, non-adherent condition embedded in Matrigel supplemented with soluble molecules, known as organoid culture, has been successfully used to culture and expand SCs and progenitors from different epithelia such as intestine, colon, gastric and liver epithelia[20–24].

The sphere-forming assay has also been used to identify CSCs in solid tumours. As in normal tissues, only a fraction of cells from human brain[25], primary colon[26] and breast cancers[27] are able to form spheres. In most cases, the cells that preferentially form spheres are also the ones that preferentially form secondary tumours on xenotransplantation[25,26,28–30]. By contrast, in mouse glioma, cells expressing low levels of inhibitor of DNA binding 1 (ID1) generated secondary tumours more efficiently than ID1^{hi} cells on transplantation but presented lower self-renewal potential in a sphere-forming assay *in vitro*[31]. These apparent discrepancies could be explained by differences in growth factors and the components of the microenvironment between xenotransplantation and *in vitro* culture assay, or it could be that these cells contribute differently to short-term and long-term tumour growth. It will be important to define in the future to what extent the ability of tumour cells to grow as spheres is directly correlated with their ability to sustain tumour growth *in vivo*.

SCs were first discovered in haematopoietic tissue[3]. The 'gold-standard' assay to assess haematopoietic SC (HSC) potential is the transplantation of a discrete population of cells into sublethally irradiated mice, allowing the assessment of the long-term renewing capacities of these cells and their differentiation potential *in vivo*[32,33]. In landmark studies, Dick and colleagues[34,35] fractionated different subpopulations of bone marrow cells from patients with acute myeloid leukaemia (AML) and demonstrated that only a minor population of AML cells expressing markers of normal HSCs presents the potential to propagate the leukaemia in immunodeficient mice[34,35]. Following these papers, many other studies have shown that populations of cells presenting a higher ability to re-form the parental tumour on transplantation into immunodeficient mice can be prospectively isolated from a great variety of solid tumours, such as breast cancer[36], brain

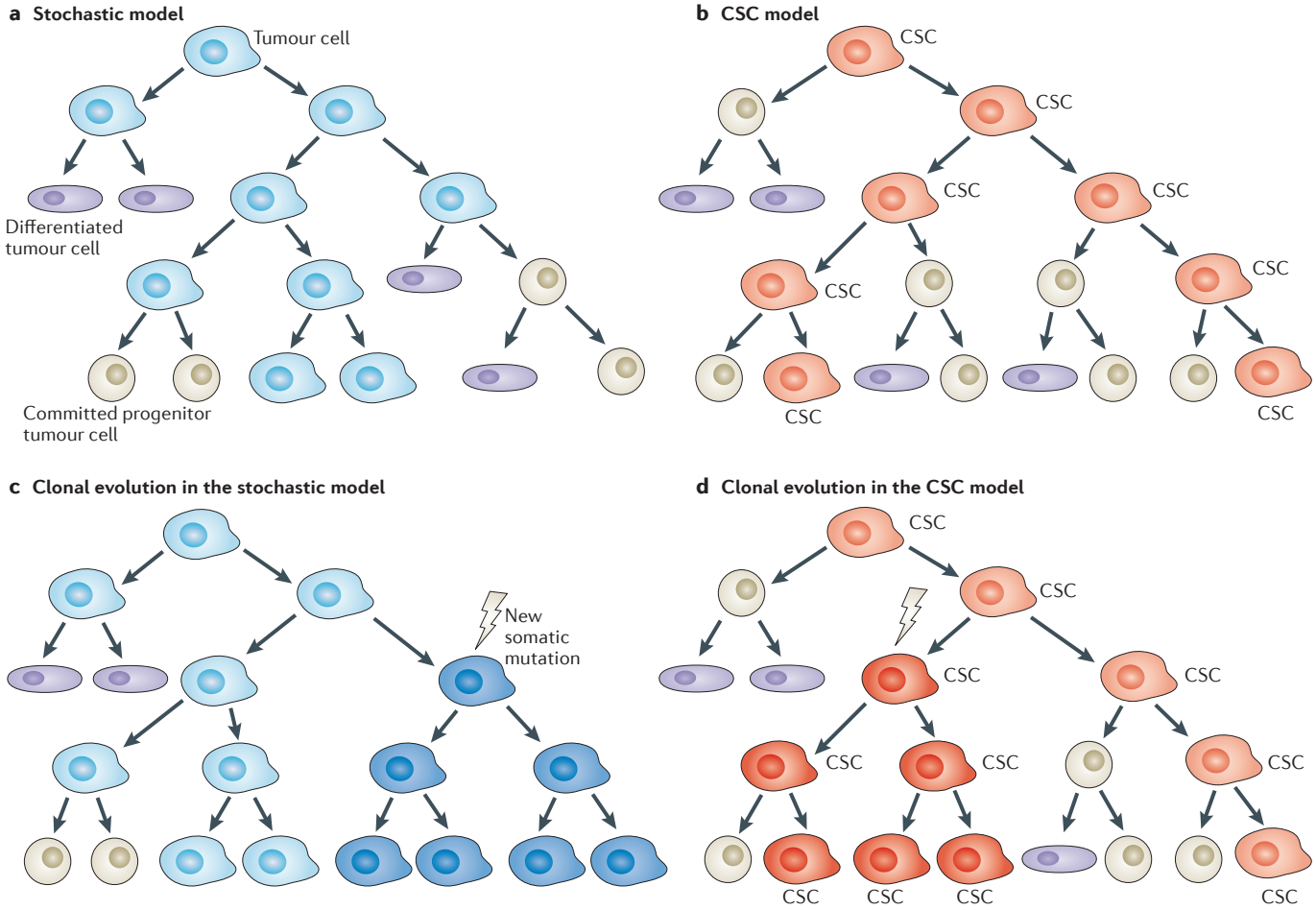

Figure 2 | **The different models of tumour growth.** **a** | In the stochastic model of tumour growth, all tumour cells are equipotent and stochastically self-renew or differentiate, leading to tumour heterogeneity. **b** | In the cancer stem cell (CSC) model of tumour growth, only a subset of tumour cells has the ability for long-term self-renewal and these cells give rise to committed progenitors with limited proliferative potential that eventually terminally differentiate. **c**,**d** | In both models, new somatic mutations can generate clonal diversity, which further increases tumour heterogeneity.

tumours[25], colorectal cancer[26,37,38], skin squamous cell carcinoma (SCC)[39], head and neck cancer[40], lung cancer[29], pancreatic cancer[41], prostate cancer[28] and ovarian cancer[30,42]. Tumour cells presenting this higher tumour-repopulating capacity have been referred to as CSCs, or as tumour-initiating cells, but the best term to describe them is probably tumour-propagating cells (TPCs), as this is the actual readout of these assays[43] (FIG. 3). It is not yet clear whether all cancers contain TPCs or what their exact frequency is within primary tumours. In many types of human tumours, TPCs have been shown to be rare, ranging from one per 100,000 to one per 1,000 cells that are capable of re-forming secondary tumours on transplantation into immunodeficient mice[44]. By contrast, Morrison and colleagues[45] demonstrated that the transplantation of melanoma cells embedded in Matrigel into more severely immunodeficient mice (NOD/severe combined immunodeficient (SCID) interleukin-2 receptor-γ (IL2Rγ)-deficient mice) enhances the frequency of TPCs by several orders of magnitude[45]. In some human melanomas, up to one cell in four is able to form a xenograft tumour[45], demonstrating that TPCs are not always rare. The relative abundance of melanoma TPCs seems to be the exception rather than the rule. More studies using the same conditions as the ones used in this study (Matrigel and NOD/SCID IL2Rγ-deficient mice) are required to assess whether the low frequency of TPCs found in many tumours is the consequence of suboptimal assays rather than due to an intrinsic inability to be propagated in immunodeficient mice.

Whether a subpopulation of melanoma cells expressing ATP-binding cassette subfamily B member 5 (ABCB5)[46], CD271 (also known as NGFR)[47] or aldehyde dehydrogenase 1A (ALDH1A)[48] is enriched for melanoma TPCs remains a matter of debate. Interestingly, a recent study showed that CD271-positive but not CD271-negative melanoma cells can re-form secondary tumours on transplantation into moderately immunodeficient (nude and NOD/SCID) mice. By contrast, in the most immunodeficient moce (NOD/SCID IL2Rγ-deficient mice), or in natural killer cell-depleted immunodeficient mice, both CD271-positive and CD271-negative tumour cell fractions are able to re-form secondary tumours. This suggests that the type, the frequency and the function of different TPC subpopulations depend on the degree of mouse immunodeficiency and whether the melanoma cells were first amplified *in vivo* or *in vitro* before their isolation by fluorescence-activated cell sorting (FACS) and transplantation into immunodeficient mice[45,47,49]. However, although natural killer cells impose a strong immunological barrier against tumour xenotransplantation, they do not change the TPC frequency in a syngeneic mouse model of SCC[50].

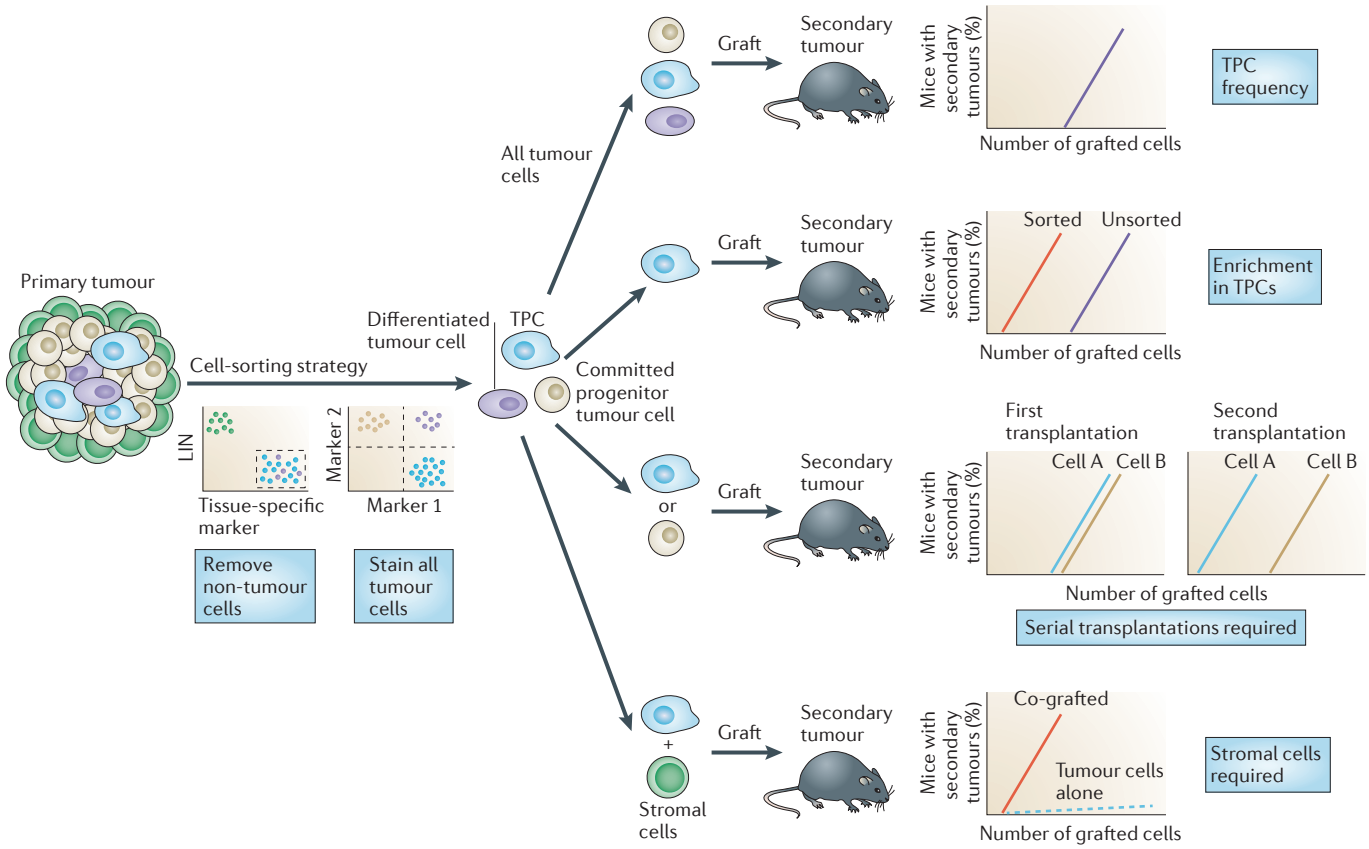

Figure 3 | **The TPC assay.** To assess tumour-propagating potential, different populations of tumour cells are isolated by flow cytometry and transplanted into immunodeficient mice. A combination of a tissue-specific and lineage-negative (LIN^-) markers allows the isolation of tumour cells, which can then be separated in different subpopulations on the basis of cell surface marker expression or enzymatic activity. Transplanting limiting dilutions of tumour cells and assessing the fraction of transplanted cells giving rise to secondary tumours allows for the estimation of tumour-propagating cell (TPC) frequency. Subsets of tumour cells giving rise to secondary tumours more frequently than unsorted tumour cells are enriched in TPCs. In certain cases, serial transplantation is required to distinguish a population of tumour cells with long-term self-renewal potential. Finally, some tumour cells may not form secondary tumours in the absence of stromal cells, owing to their strong dependence on the microenvironment. Transplantation constitutes a good approach to determine whether certain tumour cells have long-term self-renewal properties, as well as the capacity to generate all the cell types that are found in primary tumours. However, this method does not indicate whether TPCs are responsible for tumour growth within the primary tumours.

The concept of CSCs, in its most rigorous definition, implies that all cancer cells are genetically equivalent and differ only transcriptionally and/or epigenetically. However, it is now clear that, during acute lymphocytic leukaemia (ALL) progression, multiple genetically different clones of leukaemia-propagating cells coexist at the same time, and that the clonal architecture of the leukaemia is highly dynamic during disease progression, which suggests rapid multi-branching events rather than the linear accumulation of mutations[51,52]. In a related study, it was demonstrated that human ALL leukaemia-propagating cells arise from minor subclones that were present at the time of the diagnosis and that resemble the clones found during relapse after therapy, suggesting that the xenotransplantation assay may select for the most aggressive clones[53]. To assess how the evolution of genetically diverse subclones during tumour progression and response to chemotherapy affects intratumoural heterogeneity, the repopulation dynamics of 150 clones from ten human colon cancers was monitored during serial transplantation. Analyses revealed that initial tumour heterogeneity was mostly maintained over serial transplantations, although subtle genetic changes could have been overlooked. However, the long-term persistence of these clones differs with serial transplantation. Some clones are present in all serial transplants (persistent clones), other clones are lost rapidly (transient clones) or are present initially and then disappear (short-term clones). Other clones that are barely detectable in the primary tumours become predominant in the later transplants, suggesting that they are not initially highly proliferative but become activated during transplantation (dormant clones). Finally, some clones are detected initially, then not detected but then reappear (fluctuating clones). This analysis demonstrates the considerable clonal dynamics of tumour cells on xenotransplantation. These different types of clones were not equally sensitive to chemotherapy, with the slow-cycling clones being the most resistant and contributing to tumour regrowth after therapy, illustrating that the cells that fuel tumour growth can be different from the cells that are responsible for tumour relapse[54]. These results, therefore, suggest that tumour progression is associated with clonal evolution in which TPCs may compose the units of genetic evolution and selection. Moreover, the generation of monoclonal tumours by xenotransplantation of single human colon cancer cells showed that tumour heterogeneity is not exclusively the consequence of genetic heterogeneity and is partially explained by the differentiation potential of individual tumour cells[55].

Self-renewal in adult SCs and CSCs

Different assays have been developed to monitor the self-renewal of SCs and CSCs. Serial passages of colonies or spheres *in vitro*, as well as serial transplantation of tumour cells, are frequently used to assess the long-term self-renewal capacities of SCs and CSCs. Similarly to the assessment of the long-term renewal properties of HSCs[32,33], serial transplantation of tumour cell populations is required to assess the long-term renewal properties of tumour cells, as secondary tumours often reach the size that requires ending the experiment only a few weeks after their transplantation, precluding the assessment of their long-term renewal potential. In mouse SCC, TPCs are not enriched in primary tumour cells expressing high or low levels of the cell surface marker CD34 (REFS 50,56). However, TPC frequency increased in $CD34^{hi}$ and decreased in $CD34^{low}$ cells during serial transplantations[50] (FIG. 3). Likewise, spinocerebellar ataxia type 1 (SCA1; also known as ATXN1)-positive cells in mouse lung adenocarcinoma are enriched in TPCs that can be serially grafted, whereas SCA1-negative cells only give rise to small secondary tumours that cannot be serially transplanted[57].

The genetic lineage-tracing approach has become the gold-standard assay to assess the fate and long-term renewal potential of epithelial SCs in their natural environment. It has also been used successfully to define their heterogeneity, renewal and differentiation potential in many tissues during development, homeostasis, repair and tumour initiation[6,58,59]. In mouse lineage-tracing experiments, transgenic animals express a drug-inducible Cre in a specific lineage together with a reporter gene. On Cre activation, the reporter gene is permanently expressed in the cells expressing the Cre and all their future progeny. The fraction of labelled cells that persist over time is indicative of the renewal potential of these cells and the different types of differentiated cells represent the differentiation potential of SCs.

Lineage tracing in the gut and the epidermis showed that labelled SCs and their progeny could persist up to 1 year following their initial labelling, demonstrating their long-term self-renewal capacities[21,60,61]. SCs in these tissues divide asymmetrically to maintain the pool of SCs and to give rise to committed progenitors with restricted renewal potential, which generate terminally differentiated cells that are lost over time. Interestingly, in both the intestine[62] and epidermis[61], clonal analysis revealed that the balance between self-renewal and differentiation is achieved at the population level but that SC fate decision occurs stochastically at the cellular level[61–64]. SCs can also undergo symmetric differentiation, leading to the loss of some SCs, meaning that SCs are not always long-lived.

Several studies have recently used lineage tracing in pre-existing tumours to assess the fate of individual tumour cells[65,66] in their native environment (FIG. 4). Our group carried out clonal labelling of skin tumours and assessed the persistence and

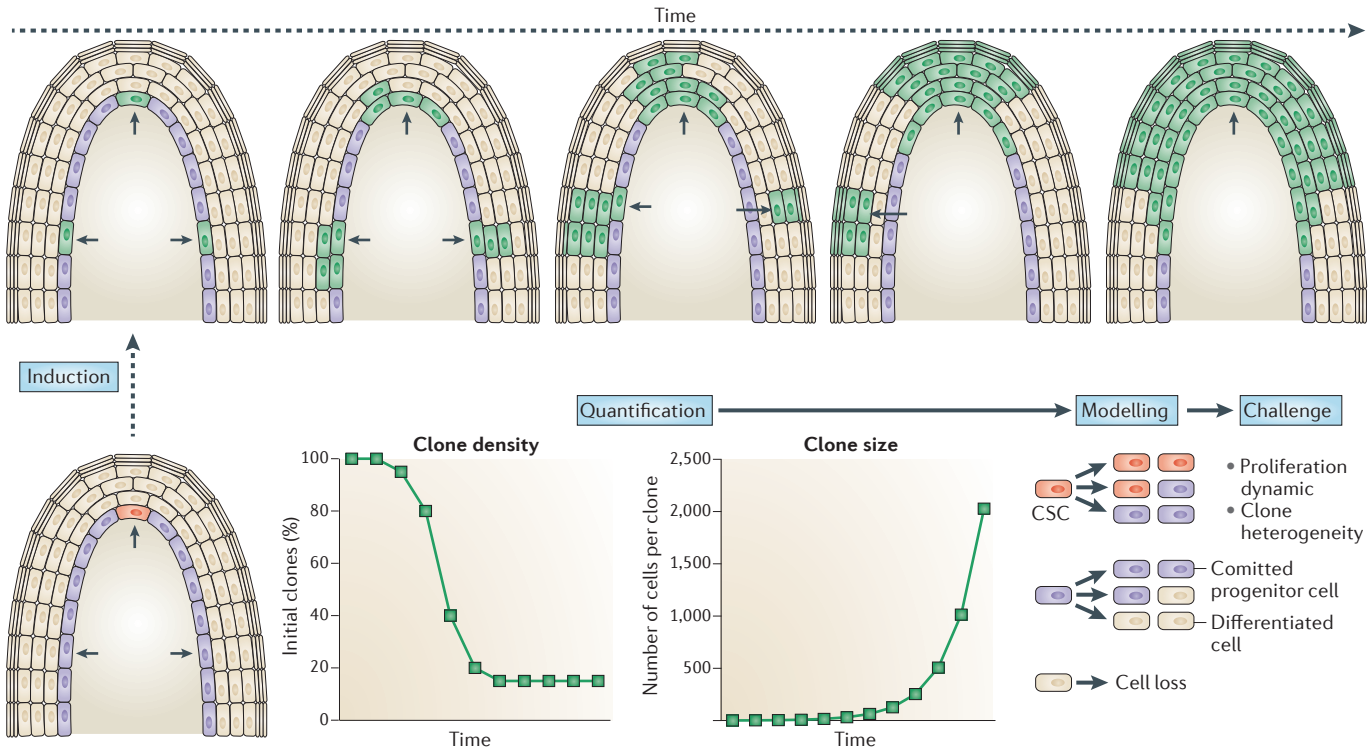

Figure 4 | **Clonal analysis to assess the mode of tumour growth.** Clonal analysis by genetic lineage-tracing experiments allows the assessment of the fate of individual tumour cells during tumour growth. In this example, a low dose of tamoxifen is administered to mice bearing skin tumours and expressing Cre recombinase fused to oestrogen receptor (Cre–ER) under the control of a promoter targeting proliferative tumour cells together with a reporter gene. Tamoxifen administration should lead to the labelling of cells (indicated by cells in green) that are sufficiently distant from each other to assess the fate of individual labelled cells over time. These quantifications allow the determination of clone survival and growth potential. Mathematical modelling of the clonal fate data can be used to predict the proliferation dynamics that lead to clonal diversity. These predictions can then be tested and challenged by new experiments to refine the model. Using this approach, several studies have already demonstrated the existence of cancer stem cells (CSCs) in different mouse models of solid tumours[65,66,74,75].

the size of labelled clones over time in benign and malignant tumours. In benign papilloma, different cell fate outcomes were observed several weeks after the initial labelling: a majority of small clones expanded transiently before being lost through terminal differentiation, while the rarer, large clones were composed of thousands of tumour cells, including proliferating and differentiated cells, which was consistent with the initial labelling of CSCs (FIG. 4). Mathematical modelling of the clonal fate data suggested that the tumour is hierarchically organized similarly to normal epidermis[61], but CSCs divide rapidly instead of being mostly quiescent (like SCs) during normal homeostasis[61,65]. This model suggests that, on division, CSCs and their progenitor cells, like normal epidermal SCs and progenitors, stochastically divide into three different fates: asymmetrical self-renewal, symmetrical renewal and symmetrical differentiation, leading to asymmetrical cell division at the population level. If this stochastic drift of SCs is happening in other types of cancers, it could potentially explain tumour heterogeneity without implying that the genetic diversity had necessarily conferred a selective advantage (neutral drift)[65]. By contrast, in malignant carcinoma, most invasive tumour cells actively proliferate and very few undergo terminal differentiation, demonstrating that malignant progression is associated with a major block of differentiation and potentially with the emergence of one type of stem or progenitor cancer cell. However, these analyses were carried out at only one time point and with a single Cre, precluding the assessment of the long-term renewal potential of these cells and the mechanisms that lead to tumour heterogeneity within these invasive cancers.

Using a technique called tumour retracing, Clevers and colleagues[66] assessed the clonogenic potential of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)-expressing cells in benign intestinal tumours[66]. Intestinal adenomas arise from activating mutations in the WNT pathway in intestinal SCs[67–69]. The authors induced intestinal adenoma by conditionally deleting adenomatous polyposis coli (*Apc*) in LGR5-expressing cells and inducing one of the four fluorescent colours of the confetti reporter construct[62]. Owing to the presence of inverted *loxP* sites in the confetti reporter construct, re-administration of tamoxifen to *Lgr5*–Cre–oestrogen receptor (ER) mice bearing adenoma marked in one colour will induce the expression of another colour within LGR5-expressing cells, allowing the tracing of the progeny of LGR5-positive cells in adenoma. These experiments demonstrated that *Lgr5*–Cre–ER marks cells that fuel tumour growth, which is consistent with the existence of CSCs expressing LGR5 in intestinal adenoma, and similar to the SCs from which these tumours arise[67,69]. These experiments suggest that in the intestine there is a continuum between the normal SCs that initiate tumour formation and the CSCs that fuel tumour growth. Ephrin type B receptor 2 (*EPHB2*) is a WNT target gene that is expressed in intestinal and colonic SCs[70]. It has been recently reported that human colon cancer cells expressing high levels of EPHB2, expressed the highest level of LGR5 and were enriched in TPCs[71]. Likewise, using antibodies against LGR5 and epithelial cell adhesion molecule (EPCAM), it is possible to isolate a population of TPCs from human primary colorectal cancers[72], suggesting that, in intestinal tumours, TPCs are equivalent to the cells that sustain tumour growth. More recently, lineage tracing using doublecortin-like and CAM kinase-like 1 (*Dclk1*)–Cre–ER demonstrated that, in the normal intestine, DCLK1 marks terminally differentiated cells, as previously suggested[73]. However, in intestinal adenoma, DCLK1 marks CSCs that, similarly to the LGR5-positive tumour cells, give rise to cells that fuel tumour growth, demonstrating that CSCs may express markers that in normal tissues characterize differentiated cells[74].

To dynamically assess the fate of tumour cells *in vivo*, Van Rheenen and colleagues[75] devised a new intravital lineage-tracing approach to image the fate of individual breast tumour cells in a living animal over time[75]. Similarly to what was reported for skin SCCs[65] and intestinal adenoma[66], during the growth of breast adenocarcinoma, dominant clones of marked cells appeared, whereas other clones regressed or disappeared, suggesting that different epithelial tumours may present qualitatively a similar mode of tumour growth.

Clearly, future studies using *in vitro* clonogenic assays, transplantation and lineage tracing are crucially needed to reconcile the different assays that are used to assess normal and tumour SC potential and to more precisely define tumour heterogeneity and proliferation dynamics of tumour cells, how the cellular hierarchy changes during tumour progression, invasion and metastasis, and whether the same cells that mediated tumour growth are responsible for resistance to therapy and tumour relapse.

Molecular features of adult SCs and CSCs

There is no universal molecular feature of adult SCs, although some markers, such as LGR5, can identify different SC populations in the digestive tract[21,60] and in the epidermis[16]. Likewise, there are also no universal markers of CSCs, although CD133 has been associated with TPCs in many different types of tumours[76]. It is sometimes assumed that the expression of such surface markers may reflect the CSC content in tumours. However, the identification of TPCs using these surface markers should always be confirmed experimentally, especially in human tumours, which may be much more heterogeneous than those in transgenic mouse models. Understanding what induces CD133 expression and identifying the co-regulated genes in tumour cells may be important to better define the transcriptional and epigenetic landscape of TPCs.

In some tumours, the genes and signalling pathways that regulate normal SC functions also function as oncogenes or regulate tumour maintenance and progression. One of the best-characterized examples is WNT–β-catenin signalling, which is essential for the maintenance and proliferation of intestinal SCs and other types of SCs[77,78]. This pathway is frequently mutated in colorectal cancers[79,80] and is required to sustain tumour growth and progression in several different types of cancers[81], including colorectal cancer, leukaemia and skin basal cell carcinoma[82,83]. During basal cell carcinoma initiation, oncogene-expressing adult interfollicular epidermis SCs are rapidly reprogrammed into an embryonic hair follicle progenitor-like fate, before progressing into carcinoma[82]. Consistent with the reprogramming of tumour cells into an embryonic-like fate, similarities between embryonic mammary SCs and the basal-like and HER2-positive breast cancer subtypes have also been described[84]. In addition, it has recently been shown that, in a mouse model of glioblastoma, oncogene expression in different cell types of the central nervous system, including SCs, astrocytes and mature neurons, is able to initiate glioblastoma and is associated with a transcriptional signature that resembles stem and progenitor cells. This is consistent with the idea that adult cells, including SCs and differentiated cells, are required to undergo reprogramming to a progenitor-like fate for tumour initiation[85]. Similarly, in the intestinal epithelium, activation of the WNT pathway together with nuclear factor-κB in normally differentiated cells of the intestine can induce

their dedifferentiation into stem cell-like cells and induce tumour initiation[86]. Taken together, these results suggest that SC markers can be expressed in tumour cells on the continuous expression or reactivation of specific signalling pathways and transcriptional networks that were active during tissue development, leading to the dedifferentiation of these cells during tumour initiation. It will be important to determine the extent to which dedifferentiation into stem cell-like fate and embryonic reprogramming are general features of tumour initiation and whether common mechanisms are involved in the tumour initiation of different cancers.

Plasticity of SCs and CSCs

Although transplantation is an excellent assay for assessing the potential of SCs, it does not assess their actual fate in their native environment. The differentiation potential of many adult SCs, such as those in the hair follicle[14,16,87], the mammary gland[88–90] and the prostate[91,92], is more restricted in lineage-tracing experiments compared with that observed in transplantation assays, suggesting that transplantation assays may mimic a regenerative state that stimulates the multilineage differentiation potential of SCs. Normal SCs also present a certain degree of plasticity *in vivo* within their natural environment during regenerative conditions. In the skin epidermis, hair plucking induces the depletion of hair follicle SCs, allowing their immediate progeny to repopulate the empty SC niche and re-acquire SC properties[93]. Similarly, in the gut, delta-like 1 (DLL1) and slow-cycling LGR5-expressing cells represent progenitors committed to the secretory and Paneth cell lineages, respectively, and usually undergo several rounds of division before terminal differentiation. However, on SC depletion (as induced by ionizing radiation) these progenitor cells can repopulate the SC niche and regain SC potential[94,95], demonstrating the important plasticity of early committed progenitors during tissue repair.

It has also been demonstrated that cell surface markers could be dynamically and reversibly expressed by tumorigenic cells[96]. In intestinal tumours, LGR5-positive and LGR5-negative cells isolated from villus-derived spheres can re-form secondary tumours on transplantation, and LGR5-negative cells can give rise to LGR5-positive tumour cells, supporting the idea that, when levels of active β-catenin are increased, villus cells can reacquire CSC properties by dedifferentiation[72]. In skin SCC, $CD34^{low}$ cells can generate $CD34^{hi}$ cells on transplantation. However, $CD34^{low}$ cells that acquire CD34 expression do not generate secondary tumours with the same efficiency as $CD34^{hi}$ cells isolated from the primary tumours[50], suggesting that the reversibility of surface marker expression does not imply that the fate of tumour cells is also fully reversible. In human breast cancers, the combination of surface markers $CD44^{hi}$ and $CD24^{-}$ marks TPCs[36]. Similarly, human breast cancer cell lines also present a $CD44^{hi}CD24^{-}$ cell population that is enriched for TPCs[97]. Interestingly, the non-TPC populations can give rise to $CD44^{hi}CD24^{-}$ cells, which suggests a stochastic state transition between these different subpopulations[98]. Future studies are needed to determine whether the stochastic state transition observed in breast tumour cell lines can also occur in primary tumours from different tissues and whether this transition is associated with increased tumorigenic potential *in vivo*.

CSCs and metastasis

Functional heterogeneity of tumour cells may also have a role in cancer progression and metastasis. Because CSCs have been operationally defined as cells that are able to re-form secondary tumours on transplantation into immunodeficient mice, it has also been suggested that CSCs could be responsible for metastasis. Interestingly, epithelial–mesenchymal transition (EMT)-associated genes, which promote tumour progression and metastasis, can also regulate normal SC and CSC properties[99] (BOX 1). In human and mouse models of medulloblastoma, it has been reported that metastases were genetically similar to each other but different from the primary tumour, suggesting that the mutations that are responsible for tumour metastasis do not necessarily give rise to a selective growth advantage in the primary tumours[100]. CXCR4 is expressed in metastases from many different cancers, including breast cancer[101,102], prostate cancer[103] and melanoma[101,104], and is therefore seen as a potential therapeutic target to treat

Box 1 | Regulation of tumour stemness by EMT

Epithelial–mesenchymal transition (EMT) is defined by biochemical changes that occur in epithelial cells that lead to the acquisition of mesenchymal properties, including the loss of cell adhesion and the acquisition of migratory properties. EMT is a physiological process that occurs during development to allow the migration of mesodermal cells during gastrulation or the delamination of the neural crest cells from the dorsal neural tube[149]. In cancer, EMT has been associated with malignant features and aggressiveness[149]. Several signals are able to trigger EMT in cancer epithelial cells, including hypoxia and factors emanating from the tumour stroma, such as transforming growth factor-β (TGFβ)[149]. These factors in turn induce the expression of different transcription factors, including TWIST1, TWIST2, Snail homologue 1 (SNAI1), SNAI2, zinc-finger E box-binding homeobox 1 (ZEB1) and ZEB2, which repress E-cadherin expression, and each has been shown to trigger EMT when ectopically expressed in epithelial cells *in vitro*[149].

EMT has not yet been directly associated with the regulation of normal SCs but many EMT markers are expressed by dermal cells with *ex vivo* SC properties, although the functional role of EMT markers in these cells remains elusive[150,151]. Several studies suggest that EMT generates cells with apparent cancer stem cell (CSC) features in different types of tumours, including breast[99,152,153], pancreatic[154] and colorectal[155] cancers. TWIST1, a basic helix–loop–helix transcription factor that is required for the formation of the head mesenchyme during embryonic development[156], has been identified as an essential regulator of breast cancer metastasis[157]. In addition, it has been shown that the overexpression of TWIST1 or SNAI1 in immortalized human mammary epithelial cell lines induces EMT, the expression of the cell surface marker CD44 and the loss of CD24 ($CD44^{hi}CD24^{low}$)[99], forming a population of cells that is reminiscent of the tumour-propagating cells previously described in breast cancer[36]. TWIST1 promotes the ability of tumour cells to form mammospheres *in vitro* and their ability to re-form secondary tumours after transplantation following overexpression of oncogenic HRAS-G12V, thus suggesting that TWIST1 regulates the acquisition of CSC properties[99]. The transcriptional changes associated with EMT resemble the gene expression profile of the $claudin^{low}$ subtypes of breast cancer[158]. TWIST1 may also prevent oncogene-induced senescence[159], possibly by promoting the expression of *BMI1* (REF. 160), a gene that prevents cellular senescence in different types of normal and cancer cells[68,161–164]. The differentiation of breast CSCs is regulated by the miR-200 microRNA cluster, which, by inhibiting *BMI1* expression, impairs mammary CSC renewal and stimulates the differentiation of these cells[165]. Although these experiments support a role for EMT and TWIST1 in the acquisition of CSC properties, the specific role of TWIST1 in regulating EMT, tumour progression and CSC functions in primary tumour cells remains elusive. It will be important to determine whether TWIST1 regulates tumour initiation, progression and stemness *in vivo* in mouse primary tumour models and to define the TWIST1 target genes that control tumour heterogeneity and progression.

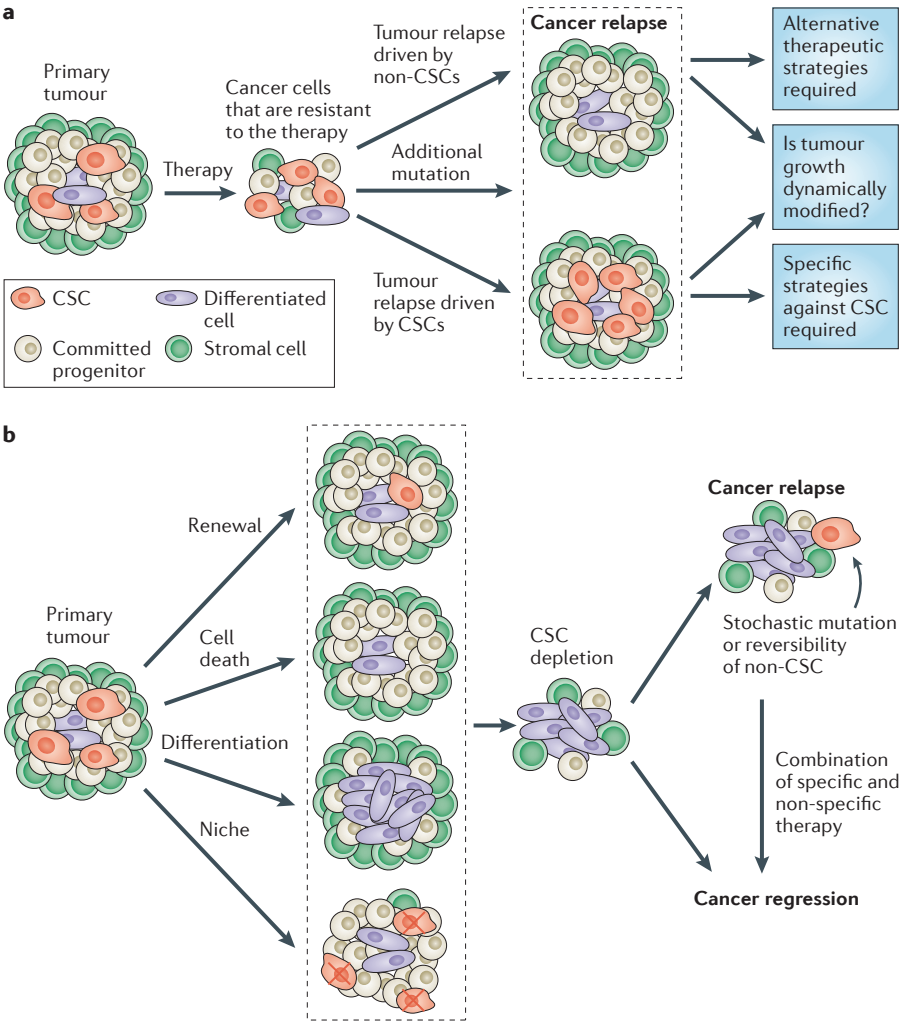

Figure 5 | **Implication of CSCs in cancer therapies and tumour relapse.** **a** | Anticancer therapies may not kill all tumour cells equally. Cancer stem cells (CSCs) that sustain tumour growth or another population of more slowly cycling tumour cells may be responsible for tumour resistance to therapies and tumour relapse. Depending on the population responsible for tumour relapse, new strategies should be designed to eradicate all tumour cells. **b** | The CSC model suggests that inhibiting CSC renewal or promoting their differentiation should induce tumour regression. Drugs could impair CSC self-renewal, induce their specific cell death, induce their differentiation or target their niche. All of these strategies would lead to the depletion of the pool of CSCs and subsequent tumour regression. However, if the CSC potential is reversible, or if newly acquired mutations confer resistance to therapy, then tumour regression would only be transient, leading to cancer relapse.

metastatic cancers[105]. However, it is still not clear whether CXCR4-expressing tumour cells that lead to metastasis represent CSCs. In human pancreatic adenocarcinoma, it has been reported that a subset of CD133-positive CSCs characterized by CXCR4 expression were more prone to form metastases. Furthermore, pharmacological inhibition of CXCR4 blocked metastasis without inhibiting tumour growth, suggesting that, in this tumour, CXCR4 expression in CSCs controls metastasis but not tumour growth[106]. Consistent with the existence of only a discrete subpopulation of tumour cells that initiate metastasis, CD26-positive human colorectal CSCs were more prone to induce liver metastasis than their CD26-negative counterparts[107]. By using genetically marked human colon cancer cells, it has been shown that primary colon cancer cells could be divided into three types: long-term TPCs that maintain tumour formation in serial xenotransplantation; transient amplifying cells that have only a limited contribution to the formation of secondary tumours; and TPCs that contribute to the formation of only the second or third serial xenografts[108]. Interestingly, the cells that were able to form metastases were almost always long-term TPCs, suggesting they could indeed be a subset of CSCs.

These studies show that tumour cells expressing certain markers, such as CXCR4 and CD26, are more prone to metastasis, but they do not provide evidence that metastasis is organized in a cellular hierarchy containing CSCs and more committed tumour progeny. It is therefore important to determine whether the cells responsible for metastasis arise from CSCs and whether metastasis presents the same clonal dynamics and hierarchy as the primary tumour.

Regulation of SCs and CSCs by their niche

Heterogeneity of SCs and CSCs could reflect the heterogeneity of their microenvironment. Tissue-specific SCs are usually localized in a specific microenvironment, called a niche, which is thought to regulate the balance between self-renewal and differentiation through either the secretion of soluble molecules or cell–cell communication[109]. Although the concept of SC niches was proposed some time ago[110], the cellular and molecular components of these niches remain generally unknown and are a matter of intense debate. It has been suggested that they are composed of specialized mesenchyme (for example, the dermal papilla for hair follicle SCs), differentiated cells (for example, Paneth cells for intestinal SCs), blood vessels (for example, HSCs) and other cell types[111]. Recently, several studies have pointed to the crucial role of endothelial cells (ECs) and the perivascular niche in the regulation of both normal SCs and CSCs. Angiogenesis has key roles in development and tissue homeostasis, as well as cancer initiation and progression, through the regulation of nutrient and oxygen delivery to the cells and regulation of the trafficking of immune cells and also through the secretion of growth factors from ECs [112].

In the central nervous system, neuronal SCs are located in close contact with ECs[113], which secrete soluble factors that maintain the self-renewal and undifferentiated phenotype of neuronal SCs[114,115]. Interestingly, CSCs in glioblastoma, a malignant brain tumour, are also localized in close contact with ECs[116]. In this model, the population of xenotransplanted CSCs is reduced on anti-vascular endothelial growth factor A (VEGFA) therapy and, conversely, culturing CSCs with ECs *in vitro* enhances their renewing capacity, suggesting that the vascular niche stimulates the maintenance of glioblastoma CSCs[116]. In SCCs, TPCs marked by CD34 (REF. 39) are also localized close to ECs[117]. Blocking angiogenesis with a VEGF receptor 2 (VEGFR2) antibody decreases microvessel density, which induces

CSC depletion and tumour shrinkage. In SCCs, VEGFA indirectly stimulates tumour stemness by stimulating the formation of its perivascular niche, and more directly through an autocrine loop involving neuropilin 1 (NRP1), a VEGFA co-receptor, which regulates symmetric CSC division and promotes tumour stemness[117]. A similar VEGFA–NRP1 autocrine loop in regulating CSCs was later reported in glioblastoma[118]. It is not clear whether NRP1 promotes tumour stemness by enhancing signalling of VEGFR1 and VEGFR2 or by directly regulating downstream signalling through its short cytoplasmic domain, as has recently been reported during endothelial development[119].

Surprisingly, although many studies point to an essential role of the perivascular niche in regulating SC functions, other studies have reported that hypoxia is an important regulator of SC function. HSCs have been proposed to reside in a vascular niche[120,121], as well as in a hypoxic niche[122,123]. Hypoxia induces the activation of the hypoxia-inducible factor (HIF) subunits HIF1α and HIF2α[124]. Deletion of *Hif1a in vivo* decreases the long-term reconstitution ability of HSCs[125] and the tumour-propagating capacity of leukaemic SCs[126,127]. Similarly, in brain and pancreatic cancers, hypoxia promotes the expansion of CD133-positive cells through the activation of HIF1α[128–130]. In ER-positive breast cancers, hypoxia also promotes the expansion of TPCs[131], whereas in ER-negative cells hypoxia decreases tumour propagation[132]. This suggests that different CSCs, depending on the cancer subtype, respond differently to hypoxia. The frequent expression of CD133 on TPCs may therefore reflect their common localization in a hypoxic environment *in vivo* or HIF1 activation under normoxic conditions[133]. The importance of the perivascular niche on the one hand and the role of hypoxia on the other hand in regulating CSCs may seem paradoxical at first glance. However, ECs in the tumour microenvironment, and possibly in the normal SC niche, might not function normally, which would lead to the generation of a hypoxic microenvironment[134].

Other cell types, such as fibroblasts[135,136], may also regulate CSC functions, but the mechanisms of these cellular interactions remain poorly understood. Different studies have recently implicated WNT signalling in the communication between CSCs and their microenvironment, which regulates their proliferation, metastasis and resistance to therapy. In colorectal cancers, tumour cells are closely associated with myofibroblasts, which secrete different factors, including hepatocyte growth factor, that stimulate WNT activation, which in turn promotes tumour stemness and CSC clonogenicity[136]. In mouse mammary tumour virus (MMTV)–polyoma middle T antigen (PyMT)-transgenic mice, mammary tumours spontaneously metastasize to the lung and periostin is expressed by lung fibroblasts, which enhances WNT signalling in circulating mammary tumour cells, promoting the establishment and the development of lung metastasis[135]. Colorectal cancer cells express high levels of transforming growth factor-β (TGFβ) but contain mutations that inactivate transduction of the TGFβ signalling pathway in cancer cells. These seemingly contradictory findings can be explained by the importance of TGFβ signalling in stromal cells (to stimulate organ colonization by circulating tumour cells), whereas inactivating mutations in the TGFβ pathway alleviate the cytostatic effect of TGFβ signalling in tumour cells, providing survival advantage to metastatic cells[137].

More studies are needed to further define the cellular and molecular components of the niche that are essential for tumour propagation, how the different cell types presented in the tumour stroma contribute to the establishment of CSC properties, and how the different immune cells inhibit and/or promote CSC functions and shape clonal evolution.

Resistance of CSCs to therapy

Normal mouse adult hair follicle SCs and HSCs are more resistant to DNA damage-induced cell death than their more committed progeny, leading to a short-term survival advantage at the expense of the long-term maintenance of their genomic integrity[138,139]. These tissue-specific SCs repair DNA damage faster and express higher levels of anti-apoptotic molecules than the more committed cell types. There is a general assumption that CSCs, like their normal SC counterparts, are resistant to chemotherapy and/or radiotherapy and are therefore responsible for tumour relapse after therapy. Consistent with this idea, TPCs are enriched in xenografts following chemotherapy of colorectal cancer[140] and after radiotherapy of glioma[141] and breast cancer[142]. In glioma, CSCs preferentially activate DNA-damage checkpoints so that they can repair DNA damage faster and escape ionizing radiation-induced apoptosis. Pharmacological inhibition of the checkpoint kinases CHK1 and CHK2 sensitizes the CSCs from brain tumours to radiotherapy[141]. Mammary SCs and breast CSCs are also more radioresistant than the other cell types in the tissue, owing to the expression of low levels of reactive oxygen species (ROS) mediated by the higher expression of free radical-scavenging machinery in these cells. Pharmacological inhibition of ROS scavengers in breast CSCs decreases their clonogenic potential and increases their sensitivity to radiotherapy[142].

If CSCs sustain tumour growth, killing CSCs directly, targeting CSC renewal or forcing CSCs to differentiate are different and independent means of potentially limiting tumour growth (FIG. 5). Today, the vast majority of patients with acute promyelocytic leukaemia are cured by the administration of all-*trans* retinoic acid and arsenic, which both promote the differentiation of leukaemic cells into terminally differentiated cells[143]. This demonstrates that promoting cancer cell differentiation can be an extremely efficient therapeutic approach to treat at least certain types of cancers. Consistent with the idea that inducing CSC differentiation might be therapeutically beneficial, it has been shown that bone morphogenetic protein 4 (BMP4) induces the differentiation of brain CSCs and inhibits tumour growth *in vivo* when BMP4-coated beads were orthotopically transplanted together with brain CSCs into immunodeficient mice[144]. The discovery of compounds that are able to inhibit CSC growth without depleting the pool of normal SCs is important to identify new anticancer therapies. A recent study screening a library of pharmacological compounds found several molecules that inhibit CSC growth by stimulating their differentiation without impairing normal SC functions[145].

Recently, Parada and colleagues[146] used a genetic lineage ablation approach in a mouse model of glioblastoma to identify the cells responsible for tumour relapse after chemotherapy. They identified a subset of normally quiescent cancer cells marked by nestin–Cre that were responsible for tumour relapse after therapy, and they demonstrated that depleting this population through lineage ablation increased survival by delaying tumour relapse compared with mice treated with classical chemotherapy[146]. In intestinal adenoma, lineage ablation of DCLK1-expressing cells, which mark CSCs and their progeny, results in tumour regression without affecting normal tissue homeostasis[74]. These studies therefore suggest that designing therapies that specifically target CSCs may lead to a considerable clinical benefit (FIG. 5).

Challenges ahead

Although tumours are usually heterogeneous and contain different populations of tumour cells with distinct *in vitro* clonogenic potentials and abilities to be propagated on transplantation into immunodeficient mice, to give rise to metastasis and to resist medical therapy, it is not yet clear whether these different populations of tumour cells represent a continuum in the clonal evolution of an ancestral CSC and perhaps of a normal adult SC, or whether each of them evolves independently after an early branching event. Studying the genetic and epigenetic mechanisms that control these different populations and their possible interconversion may provide some insight into these important issues.

It is generally assumed that the cells presenting the greatest tumour propagating potential would be the CSCs sustaining tumour growth *in vivo*. However, it is still unclear whether the cells that present the highest clonogenic potential in transplantation assays are the same cells that fuel tumour growth in lineage-tracing experiments. New techniques allowing the isolation and transplantation of tumour cells and at the same time carrying out genetic lineage tracing *in vivo* with the same marker will be essential to address this remaining uncertainty.

Lineage tracing and clonal analysis experiments have now provided compelling evidence that intratumoural heterogeneity is compatible with the existence of CSCs, at least in certain mouse models of cancer. It is now essential to carry out similar experiments in human cancers. These experiments will require the establishment of new methods to carry out clonal analysis in human tumours using either inducible lineage tracing or viral barcoding[147,148]. As these experiments cannot be done in primary human tumours, they will still require xenotransplantation assays.

Xenotransplantation of human tumour cells presents some inherent drawbacks, such as the lack of signalling capacities of some mouse growth factors to human cancer cells and the residual immunity against human cells, which differs from the normal immune response against tumour antigens in an autologous setting. It will therefore be important to develop humanized mouse models to better mimic the microenvironment found in primary human cancers, including the reconstitution of the immune system with autologous bone marrow.

It is important to better understand the mechanisms involved in cancer resistance to therapy and to determine whether the cells responsible for cancer relapse are similar to the ones that fuel tumour growth, as well as whether the proliferation dynamics of tumour relapse resemble those of sustaining tumour growth. Lineage ablation constitutes a good approach to confirm a putative cellular hierarchy in tumours but also to mimic the consequences of a specific therapy against CSCs. This will help to determine whether the fate of non-CSCs is modified on the loss of CSCs and whether they can convert into new CSCs that sustain tumour growth and drive cancer relapse. Finally, in the area of personalized medicine, the development of novel assays to predict human tumour response to therapy will be helpful to choose the most appropriate treatment, increasing our chance to treat cancer more successfully.

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doi:10.1038/nrc3597

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Acknowledgements
We apologize for the works not cited in the text owing to space restriction. B.B. is a chargé de recherche of the Fonds de la Recherche Scientifique (FNRS). C.B. is an investigator of Walloon Excellence in Lifesciences and Biotechnology. C.B. is supported by the FNRS, the Interuniversity Attraction Poles programme, the ARC programme (Action de Recherche Concertée), a research grant from the Fondation Contre le Cancer, the Université Libre de Bruxelles fondation, the fond Gaston Ithier, and a starting grant from the European Research Council.

Competing interests statement
The authors declare no competing financial interests.