Abstract | Since their identification in 1994, cancer stem cells (CSCs) have been objects of intensive study. Their properties and mechanisms of formation have become a major focus of current cancer research, in part because of their enhanced ability to initiate and drive tumour growth and their intrinsic resistance to conventional therapeutics. The discovery that activation of the epithelial-to-mesenchymal transition (EMT) programme in carcinoma cells can give rise to cells with stem-like properties has provided one possible mechanism explaining how CSCs arise and presents a possible avenue for their therapeutic manipulation. Here we address recent developments in CSC research, focusing on carcinomas that are able to undergo EMT. We discuss the signalling pathways that create these cells, cell-intrinsic mechanisms that could be exploited for selective elimination or induction of their differentiation, and the role of the tumour microenvironment in sustaining them. Finally, we propose ways to use our current knowledge of the complex biology of CSCs to design novel therapies to eliminate them.

The CSC paradigm

A semantic dispute? The cancer stem cell (CSC) hypothesis posits the existence of subpopulations of neoplastic cells within a tumour that have an elevated ability to seed new tumours upon experimental implantation in appropriate animal hosts. Implicit in this power is the ability of such cells to divide asymmetrically, yielding daughter cells that remain as CSCs (the trait of self-renewal) as well as daughter cells that differentiate into the neoplastic cells forming the bulk of the tumour. The reference to this notion as a hypothesis stems from the early days of CSC research, when their existence was the object of scepticism and intense debate. However, in light of a large body of supporting research reported in recent years, the existence of multiple subpopulations within a tumour with distinct tumour-initiating powers is no longer a matter of speculation and hypothesis. Accordingly, the use of the term ‘cancer stem cell paradigm’ now seems to be more appropriate (see BOX 1 for a brief history of CSC research, and FIG. 1 summarizing the rationale for targeting CSCs).

Beyond debates about the existence of CSCs are yet others surrounding the terms used to describe these cells. Participants of The 2011 Working Conference on CSCs¹ have outlined guidelines on how to define these cells depending on the biological system in which they are being studied. Initially used by Edmund Beecher Wilson in 1896 (REF. 2), the term ‘stem cell’ was associated with normal development for almost a century before its use in the context of cancer in the late 1980s³,⁴. The century-long use of the term ‘stem cell’ in the context of normal embryonic and adult development precluded, in the minds of some, its use in other contexts, notably those associated with neoplasia. Although normal stem cells often exhibit an ability to differentiate into multiple distinct cell types, to date most CSCs are not known to differentiate into more than a single cell type — the cells composing the bulk of the tumour. However, evidence for the multi-lineage differentiation potential of CSCs has been reported in colon carcinomas and leukaemias³,⁴, providing further basis for the notion that they reside at the apex of a hierarchy and that they possess core traits of self-renewal and differentiation, as do normal stem cells.

Although the phenotypes of normal stem cells seem to be fixed and therefore easier to identify, the phenotypes of CSCs are complex, variable from one tumour to another, and often affected by the abnormalities resulting from the process of neoplastic transformation; it is therefore often difficult to rigorously define CSCs by associating them with traits beyond their shared functional trait of tumour-initiating ability. Moreover, the existence of CSCs within tumours implies that cancer cells sharing a common genetic make-up can nevertheless exist in at least two alternative phenotypic states: as CSCs and as non-CSCs.

Intratumoural heterogeneity and CSCs — two sides to the same coin? The existence of several forms of intratumoural heterogeneity has been discussed in detail elsewhere⁵. Taking breast cancer as an example, exome and whole-genome sequencing efforts have shown that the majority of these tumours have more than one driver mutation, with a large proportion of abnormalities affecting so-called passenger genes⁶. The presence of such a large number of genetic abnormalities could lead to the occurrence of different subpopulations within the tumour, each possessing different combinations of genetically derived predispositions for growth, survival and dominance in the tumour microenvironment. Moreover, single-cell sequencing has enabled the identification of distinct subpopulations within human breast cancer samples, identifying one of these as the dominant, metastasis-seeding population⁷. In addition to their contributions to the intratumoural heterogeneity of genetic alterations, cancer cells carry heritable epigenetic alterations, which may serve equally well to generate phenotypically distinct subpopulations within tumours.

The existence of CSCs represents an entirely distinct dimension of intratumoural heterogeneity. Thus, each of the above-described subpopulations of carcinoma cells within a tumour may carry its own...
The field of cancer stem cell (CSC) research was first launched by the experimental demonstration that certain minority subpopulations of primary human acute myeloid leukaemias (AMLs) could propagate disease in immunodeficient mouse hosts at higher frequencies than the bulk populations of leukaemic cells forming these neoplasms\textsuperscript{146}. These leukaemia-initiating cells were studied when they were grown as xenografts in severe combined immunodeficient (SCID) mouse hosts and, as such, were termed SCID leukaemia-initiating cells. They were found to exhibit cell-surface antigen marker phenotypes similar to those of normal SCID-repopulating cells, implying that the cell of origin for this disease was closely allied to a haematopoietic stem cell\textsuperscript{147}. Over the ensuing decade, these observations led to searches for corresponding CSCs in solid tumours, resulting in the discovery of such populations in breast\textsuperscript{102,107}, brain\textsuperscript{13}, prostate\textsuperscript{135}, ovarian\textsuperscript{148}, colon\textsuperscript{150,151,152}, liver\textsuperscript{151,152}, lung\textsuperscript{12} and pancreatic tumours\textsuperscript{13}. In each case, these CSC subpopulations, which usually exist as minority subpopulations within tumours, have been defined operationally by their elevated tumour-initiating ability relative to that of corresponding majority populations of neoplastic cells in various tumours (Fig. 1).

Despite repeated successes in identifying such tumour-initiating subpopulations of cells within individual tumours, there has been substantial scepticism and controversy arising from the fact that these repopulation studies were carried out in immunocompromised mice, which lack an adaptive immune system and whose tissue microenvironments may therefore differ substantially from those present in humans\textsuperscript{153}. Additionally, studies in several cancers, such as AML and melanoma, have shown that CSCs need not be rare subpopulations and could be as frequent as one in four cells\textsuperscript{154,155}. It is now well recognized that certain types of cancer, such as melanoma, do not closely follow the CSC paradigm developed from studying carcinomas, whereas many others, such as breast cancer, do indeed do so. Moreover, studies in genetically engineered mouse models (GEMMs) have demonstrated the presence of CSCs in certain leukaemias and breast cancer, providing direct support for the CSC model in syngeneic models\textsuperscript{155,156}.

More recently, three lineage-tracing studies have provided unequivocal evidence of the existence of CSCs in syngeneic models of autochthonously arising tumours. One group used a GEMM that expresses the \Delta T\textsubscript{KS}-IRES-GFP transgene driven by the promoter of the Nestin gene that is normally expressed in neural stem cells; this transgene ensured expression of both thymidine kinase (TK) and green fluorescent proteins (GFPs) in stem cells. Upon crossing these TK-IRES-GFP mice with a GEMM strain engineered to develop recombinase, via administration of tamoxifen, enables single LGR5\textsuperscript{+} intestinal stem cells. These mice were crossed with multicolour Cre reporter mice in which the activation of Cre recombinase, via administration of tamoxifen, enables single LGR5\textsuperscript{+} stem cells to randomly adopt one of four alternative fluorescent labels. This led to the formation of single-coloured tumours that consisted of LGR5\textsuperscript{+} tumour-initiating population. A different strategy was followed by a second group, which used a GEMM that expresses yellow fluorescent protein (YFP) in the keratin-14-expressing cells of the basal layer of the skin epidermis, doing so conditionally in response to Cre-mediated recombination. This labelling strategy was used to trace carcinogen-induced squamous skin papillomas, revealing that the bulk of such tumours had limited proliferative capacity. However, ~20% of cells possessed the capacity for long-term propagation and were capable of giving rise to progeny that constituted the majority of the tumour. This indicated that a subset of the tumour cells — the CSCs — were capable of generating the bulk of the tumour, ostensibly by undergoing asymmetric division. During the transition from a benign papilloma to a more malignant squamous cell carcinoma, an increase in the numbers of these long-term replying CSCs was accompanied by a concurrent decrease in the number of differentiated cells. The increase in the proportion of CSCs correlates with the increased aggressiveness of the tumour upon transition to a full-blown carcinoma\textsuperscript{157}.

A small population of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5\textsuperscript{+}) cells residing in the intestinal crypt has been found to have the potential to divide both symmetrically and asymmetrically and has previously been identified as the stem cell population in this organ\textsuperscript{158}. A third group used LGR5\textsuperscript{+} adenosomatous polyposis coli (Apc)-mutant mice; in these mice, the Apc mutation leads to aberrant activation of the WNT pathway specifically in LGR5\textsuperscript{-}expressing intestinal stem cells. These mice were crossed with multicolour Cre reporter mice in which the activation of Cre recombinase, via administration of tamoxifen, enables single LGR5\textsuperscript{+} stem cells to randomly adopt one of four alternative fluorescent labels. This led to the formation of single-coloured tumours that consisted of several cell types, indicative of the presence of individual LGR5\textsuperscript{+} CSCs, each of which could give rise to a tumour containing several distinct cell types. Additionally, when a second pulse of Cre activation was induced by administering a low dose of tamoxifen, a few of the LGR5\textsuperscript{+} CSCs changed to a different colour. This gave rise to a stream of cells in the newly displayed colour, showing that these CSCs were consistently a source that could replenish the bulk of cells in each of the observed adenomas\textsuperscript{159}.

These studies have verified the existence of CSCs in three different tumour models, eliminating major doubts about the existence of such populations within the syngeneic tumour microenvironments of autochthonously arising tumours. Moreover, these studies provided compelling evidence that such CSCs adhere to the stem cell model by self-renewing and simultaneously generating progenitors that have lost their stemness, and thus proceed to form the bulk of a tumour.
studies of normal tissues have uncovered a series of these antigens that have subsequently proved to be useful for segregating CSCs from non-CSCs within tumours.

In addition to such cell-surface markers, certain intracellular proteins have been useful for studying normal stem cells and CSCs. Aldehyde dehydrogenase 1 (ALDH1) is a protein that has been used to mark CSCs in several cancers, including leukemias and carcinomas of the breast, colon, liver, lung, and pancreas, among others. The ‘side population’ that is capable of efflux of the Hoechst 33342 dye represents another non-cell-surface marker that has been used for the isolation of normal stem cells and CSCs. The most commonly used markers and marker combinations are summarized in Table 1.

The similarity of antigen display by normal tissue stem cells and the CSCs from corresponding neoplastic tissues seems to lend further support to the notion that CSCs are, at least in certain respects, bona fide stem cells. According to some, this seems to indicate that CSCs are derived directly from the stem cells in the normal tissue of origin of a tumour. However, as discussed in Box 2, alternative hypotheses describing the origins of CSCs must also be considered.

Despite the growing list of CSC markers, it has been reported that several of these are not uniformly useful in identifying CSCs. For example, although the CD44+CD24− profile was used in early studies of breast CSCs, the authors report that not all breast cancer cell populations could be stratified using this set of markers. In fact, several of these markers, including CD44, CD90 and CD34, have roles in cell adhesion and attachment and have been thought to favour the survival of the xenografted cells, which argues that the procedure of xenotransplantation may inadvertently select for the outgrowth of cells expressing these cell-surface proteins. This suggests that, in the long run, it may be more advantageous to use markers that function physiologically to support the CSC phenotype, thereby ensuring close linkage between marker display and residence in the CSC state. For instance, components of the signalling pathways that have an essential role in the biology of colorectal CSCs, such as those driven by canonical WNT signalling, may eventually yield highly specific and thus highly useful CSC markers. A more detailed discussion of CSC markers has been recently published.

**EMT and the stem cell state.** The cellular biological programme termed epithelial-to-mesenchymal transition (EMT) was initially studied because of its crucial roles in many of the cell-type interconversions underlying organogenesis during normal development. During passage through an EMT, epithelial cells lose their differentiated characteristics of cell–cell adhesion and lack of motility, instead acquiring the traits of mesenchymal cells that confer on them migratory and invasive powers along with an elevated resistance to apoptosis. In the context of carcinoma pathogenesis, EMT has been increasingly linked to the ability of carcinoma cells to invade locally and disseminate to distant anatomical sites, where they may then initiate metastases.

In general, activation of an EMT programme in both normal and neoplastic cells appears to require heterotypic signalling between these cells and neighbouring...
stomal cells. Thus, stromal signals, largely in the form of secreted factors, are released by various stromal cell types and impinge on nearby epithelial cells, resulting in the induction of intracellular signalling cascades in the latter. These pathways lead to the expression of transcription factors that orchestrate the EMT programme (known as EMT-TFs) and regulate various target genes whose expression ultimately results in the acquisition of mesenchymal cell traits.

There is rapidly accumulating evidence from diverse laboratories showing that the EMT process contributes to the progression of several carcinoma types. The EMT programme has also been shown to result in the generation of epithelial cells that have stem-like properties. This appears to be true for both normal and neoplastic mammary epithelial stem cells, the latter representing cells that exhibit CSC-like properties. Indeed, currently available evidence is compatible with the notion that epithelial cells in the normal mammary gland use components of the EMT programme as the main route for entering into the stem cell state. It remains to be seen whether other epithelial tissues similarly rely on versions of the EMT programme to generate their own normal and, by extension, neoplastic stem cells. It appears that, depending on the EMT-TFs involved, epithelial cells may enter into the stem cell state, the mesenchymal state, or both. Accumulating evidence, as discussed in more detail below, suggests that normal and neoplastic stem cells arising in epithelial tissues generally exhibit a mixture of epithelial and mesenchymal traits, which indicates that they have advanced only partially through an EMT programme.

**Signalling pathways characteristic of EMT-induced CSCs.** As EMT is a key programme for generating CSCs, it has become important to elucidate the signalling pathways that are responsible for the activation of this programme and for the maintenance of cells in the resulting mesenchymal (or quasi-mesenchymal) state. In the case of carcinomas the EMT programme is often and perhaps invariably induced through the convergence of various signals deriving from the tumour stroma, including extracellular

### Table 1 | Commonly used CSC markers, their expression and function in normal tissue

| Marker | Expression in normal tissue | Normal function | Reported malignancies |
|--------|-----------------------------|-----------------|----------------------|
| CD34   | Haematopoietic stem and progenitor cells, endothelial cells | Regulator of cell adhesion | Haematological malignancies in combination with CD38 (REF. 146) |
| CD38   | Haematopoietic cells, skeletal and heart muscle, proximal convoluted tubules of kidney, normal adult prostate | Ectoenzyme involved in signal transduction, calcium signalling and cell adhesion | Haematological malignancies in combination with CD34 (REF. 146) |
| CD44   | Leukocytes, epithelial cells, endothelial cells, mesenchymal cells | Varied functions including cell adhesion and migration, cell–cell interactions, cell signalling, leukocyte attachment and rolling | Breast cancer in combination with CD24 (REF. 28); colon cancer in combination with EpCAM; gastric cancer; head and neck cancer; liver cancer in combination with CD90 (REF. 152); ovarian cancer in combination with KIT; pancreatic cancer in combination with EpCAM and CD24 (REF. 176); prostate cancer in combination with integrin α, β, and CD133 (REF. 14) |
| CD24   | B cells, follicular dendritic cells, granulocytes, epithelial cells | B cell proliferation and maturation; function in other tissue is poorly understood | Breast cancer; gastric cancer in combination with CD44 (REF. 178); pancreatic cancer in combination with EpCAM and CD44 (REF. 176) |
| CD90   | Fetal liver cells and thymocytes, haematopoietic stem and progenitor cells, mesenchymal stromal cells, activated endothelial cells, neuronal cells | Regulation of cell adhesion, signal transduction in T cells | Brain; liver and lung tumours |
| CD133  | Haematopoietic stem and progenitor cells, endothelial progenitor cells, fetal neural stem cells, renal stem cells, prostate stem cells | Poorly understood | Brain; colon cancer; endometrial cancer; liver cancer; lung cancer with ABCG2 or CXCR4; ovarian cancer; pancreatic cancer in combination with CXCR4; ovarian cancer; breast cancer; colon cancer in combination with CD133 (REF. 22); melanoma; pancreatic tumours |
| ALDH   | Epithelial cells of the oesophagus, stomach, intestine, colon, liver, mammary gland and pancreas, Endocrine cells of the adrenal, thyroid and salivary glands, haematopoietic cells | Conversion of aldehydes generated by metabolic processes into carboxylic acids, Involved in ester hydrolysis and functions as an antioxidant, Involved in retinoic acid signalling | Breast cancer; colon cancer; head and neck cancer; liver cancer in combination with CD133 (REF. 28); gastrointestinal tumours; gliomas; hepatocellular carcinomas; lung cancer; thyroid cancer |
| Hoechst 33342 dye exclusion ('side population') | Not an endogenous marker, but allows for the selection of cells that efflux the dye via the ABC transporter. In normal tissues, these include haematopoietic and pulmonary stem cells, mammary and neural stem/progenitor cells, as well as cardiac, hepatic, keratinocyte progenitors | N/A | Astrocytomas; gastrointestinal tumours; gliomas; hepatocellular carcinomas; lung cancer; thyroid cancer |

ALDH, aldehyde dehydrogenase; ABC, ATP-binding cassette; CSC, cancer stem cell; CXCR4, CXC-chemokine receptor 4; EpCAM, epithelial cell adhesion molecule.
matrix components (such as collagen) and secreted factors such as transforming growth factor-β (TGFβ) as well as canonical and non-canonical WNTs. Such signalling cascades induce the expression of the EMT-TFs mentioned above, which include members of the SNAIL, TWIST and ZEB (zinc finger E-box-binding homeobox) family of proteins, among others. These proteins are responsible for orchestrating the gene expression programmes that activate effectors of the EMT phenotype, through the repression of epithelial genes and the activation of mesenchymal genes.

The TGFβ pathway is the first and best-studied signalling cascade operating to induce the EMT programme in various epithelial tissue types. Binding of the TGFβ ligand induces dimerization of the type 1 and type 2 TGFβ receptors, leading in turn to the phosphorylation of SMAD2 and SMAD3, which form a complex with SMAD4; once formed, the resulting transcription factor complex migrates to the nucleus, where it can induce — among other responses — a transcriptional programme that mediates the acquisition of mesenchymal properties and suppression of epithelial traits. In the context of cancer, this canonical TGFβ pathway is also known to collaborate with several other pathways, including the extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK), WNT–β-catenin and phosphoinositide 3-kinase (PI3K) signalling pathways, to promote the mesenchymal and migratory properties of cancer cells. Similarly, both canonical (WNT–β-catenin) and non-canonical (WNT-PCP (planar cell polarity)–JNK (JUN N-terminal kinase)) types of WNT signalling are responsible for the induction of an EMT and stem-like properties in various tissue types.

Recent studies have unveiled a novel collaboration among the aforementioned pathways in the induction of an EMT; this signalling operates through paracrine signals that originate ostensibly in the tumour microenvironment. In particular, secreted factors such as transforming growth factor-β (TGFβ) and Gremlin by epithelial cells inhibit autocrine TGFβ signalling, and the production of Dickkopf-related protein 1 (DKK1) and secreted Frizzled-related protein (SFRP) by epithelial cells inhibits autocrine WNT signalling, protecting the epithelial cells from inadvertent activation of signalling that would lead to activation of the EMT programme. Conversely, the shutdown of these secreted inhibitors in mesenchymal cells enables autocrine TGFβ and WNT signalling, permitting the activation of mesenchymal gene expression programmes. Given the apparent close connection between the EMT programme and the CSC state, these dynamics would seem to also apply to entrance into and out of this phenotypic state. Other signalling pathways that are implicated in the induction and maintenance of CSC traits include prostaglandin E2, Hedgehog and Notch and platelet-derived growth factor receptor (PDGFR); a summary of these pathways is presented in FIG. 2.

The interplay between such autocrine signals produced by carcinoma cells and paracrine signals arising in the tumour microenvironment presumably creates a complex array of cues that dictate the extent of the epithelial (non-CSC) and mesenchymal (CSC) characteristics displayed by carcinoma cells. The possibility that these signals can function in an essentially unlimited number of combinations implies the existence of multiple distinct phenotypic states between the fully differentiated, strictly epithelial state and the fully mesenchymal state — with these two states representing the extremes of the EMT programme.

In truth, the extent of the epithelial versus mesenchymal polarization that carcinoma cells undergo within actual human tumours is poorly resolved at present. It seems increasingly likely that carcinoma cells that have activated an EMT programme usually enter into a state in which certain epithelial markers are retained while new mesenchymal markers are acquired, resulting in what is...
often termed a ‘partial EMT’. Accordingly, cells that have passed entirely through an EMT programme and have thus undergone a ‘complete EMT’ resemble transdifferentiated cells of the mesenchymal mesodermal lineage. These cells lose the epithelial versus mesenchymal plasticity that is required for the expression of tumour-initiating properties, traits that are retained by cells that undergo a partial EMT. Moreover, bona fide epithelial stem cells — both normal and neoplastic — would seem to arise from cells that have such mixed epithelial and mesenchymal properties.

**Resistance to conventional drugs.**
Chemotherapy and radiotherapy have been the treatments of choice for the past half-century, often affording remarkable reductions in tumour burden. As briefly mentioned above, induction of an EMT leads to the acquisition of resistance to both forms of therapy — a phenomenon that has been documented in the greatest detail in breast and ovarian cancers.

Chemoresistance has also been shown to be higher in tumours that harbour a gene signature that is indicative of desmoplastic or reactive stroma, which is consistent with the notion that signals secreted by a reactive stroma have a major role in the induction of an EMT. In light of the complex regulation of the CSC state and its maintenance, how might one utilize the current knowledge of the CSC state and its maintenance, specifically target this treatment-resistant subpopulation?

At present, we possess only an incomplete understanding of the actual biochemical and cell-physiological mechanisms underlying the intrinsic chemoresistance and radio-resistance of tumour cells that have passed, even partially, through an EMT. Moreover,
resistance to cytotoxic treatments may also be attributable to the lower proliferative rate that results from the acquisition of mesenchymal properties \(^{50-54}\). Indeed, CSCs from a variety of tumours have been shown to be slow-cycling and to exhibit an increased level of quiescence compared to most of the populations of cancer cells within certain tumours \(^{26,25}\). Additionally, the resistance to chemotherapy in normal stem cells has been attributed to the high-level expression of anti-apoptotic proteins \(^{49}\) and to ATP-binding cassette (ABC) transporters that are capable of inducing the efflux of drugs (when treated with the Hoechst 33342 dye, they create a ‘side population’, which can be isolated using fluorescence-activated cell sorting (FACS) fractionation of tumour cell populations \(^{37,40,66}\); these mechanisms could also operate to confer similar properties on CSCs.

A recent study using a genetically engineered mouse model of glioblastoma development has shown that a quiescent population of tumour cells survives treatment with temozolomide and regenerates the tumour by differentiating into populations of highly proliferative cells \(^{37}\). This finding demonstrates directly that the CSCs in this tumour exhibit elevated resistance to chemotherapy, and that purely cytotoxic treatment regimens that target cycling cells are bound to fail unless they are accompanied by a targeted therapy that specifically targets these small, phenotypically distinct subpopulations (FIG. 1).

**Therapeutic targeting of CSCs**

**Understanding of CSC-dependent signalling pathways.** The identification and characterization of CSCs has revealed the need for specific molecular therapies that target the key signalling pathways supporting these cells and their residence in the CSC state. As described above, CSCs and normal stem cells share a number of properties. This explains why signalling pathways, such as those activated by WNT, TGFβ, Notch and Hedgehog — all of which are known to be essential for the self-renewal properties of normal adult stem cells \(^{38,53,63,64}\) — are emerging as attractive targets owing to the fact that their inactivation may allow the elimination of CSCs.

Studies of CSCs and the EMT programme have led to a preliminary understanding of the signalling pathways that are preferentially used by these cells, examples of which are illustrated in FIG. 2. From these studies it has become evident that these pathways are highly context dependent and several of them may actively collaborate to maintain residence in the CSC state, one example being the aforementioned activation of both the TGFβ and WNT signalling pathways in the maintenance of mammary CSCs \(^{29}\). These extracellular signalling channels may offer opportunities for interdicting these pathways in the extracellular space through, for example, neutralizing antibodies.

**Screens to identify novel targeted therapeutics.** Pharmacology has been profoundly changed by the ability to screen large, complex chemical compound libraries in order to identify chemical species that target specific proteins within cells. In the case of CSCs, chemical screening for agents that specifically target these cells has been a challenge owing to their rarity and the inability to propagate in culture CSC populations that have been isolated by flow cytometry. One recent strategy to circumvent this hurdle has involved the screening of mammary epithelial cells that have been forced experimentally to undergo an EMT and have thus acquired certain CSC characteristics, including an increased tumour-initiating ability in vivo \(^{39,40}\).

One group has carried out a 16,000-compound library screen to identify compounds that could preferentially kill EMT-induced CSCs; these CSCs were derived through knockdown of E-cadherin, an alteration that is known to favour activation of the EMT programme \(^{37}\). Through this screen, it was shown that pre-treatment of CSCs with salinomycin resulted in an approximately 100-fold decrease in tumour-seeding ability relative to the conventional agent, paclitaxel. Similar screens have subsequently been carried out by others to identify compounds that preferentially target glioblastoma CSCs \(^{40}\), ovarian CSCs \(^{51}\), breast CSCs \(^{35}\) and acute myeloid leukaemia (AML) stem cells \(^{52}\).

In principle, such screens can allow, in an unbiased way, the identification of novel modulators of cell phenotype. However, these studies also highlight that our understanding of CSCs and the pathways that these cells depend on is still incomplete. Like other similar drug development strategies, such screens should be used as starting points for further functional studies that reveal, at a mechanistic level, precisely how these agents actually work. Additionally, screens such as these are carried out in two-dimensional cultures in the absence of components that would ordinarily be present in the tumour microenvironment, such as the extracellular matrix, stromal cells (including fibroblasts, myofibroblasts and immune cells), as well as endothelial cells forming microvessels. Such deficiencies must be taken into account when attempting to extrapolate the results of these screens to the observed behaviour of CSCs in vivo.

Ideally a future anti-CSC therapy, using agents such as those cited above, should eliminate the pool of cancer cells that are intrinsically resistant to conventional therapies, while a concomitantly administered conventional agent would eliminate the non-CSCs that are known to be susceptible to existing cytotoxic therapies. Importantly, elimination of the CSCs alone may not suffice to induce an acceptable, durable clinical response, as new CSCs may be generated in CSC-depleted tumours via the spontaneous dedifferentiation of non-CSCs — a consequence of cellular plasticity that enables the emergence of de novo CSCs from differentiated cells \(^{53,54}\).

**Targeting the tumour microenvironment.** Direct targeting of CSCs represents one major strategy for eliminating these cells and, consequently, the tumours that they support. However, alternative strategies have been suggested as a result of the rapidly growing information on the tumour microenvironment, particularly its role in triggering the activation of an EMT programme in carcinoma cells and the possible entrance of these cells into the CSC state. As mentioned above, heterotypic signals arising in the tumour-associated stroma are often responsible for activating this programme in nearby carcinoma cells. Prominent among the signal-emitting cells of the stroma are fibroblasts, myofibroblasts, adipocytes and mesenchymal stem cells (MSCs), infiltrating immune cells such as macrophages and neutrophils, as well as endothelial cells that make up the walls of blood vessels that extend through the tumour (BOX 3; FIG. 3). Detailed reviews of these stromal components have been previously published \(^{55,56}\). In addition, the extracellular matrix assembled by these cells has strong effects on invading carcinoma cells \(^{37}\).

In the case of colon carcinomas, the interactions between carcinoma cells and stromal cells, specifically myofibroblasts, have been shown to be important in inducing and maintaining a more stem-like state in the tumour cells \(^{46}\), which directly shows that the stroma can have a major role in the generation of CSCs. Moreover, interactions between certain classes of carcinoma cells and MSCs induce the latter to secrete prostaglandin E\(_2\) (PGE\(_2\)), which is then responsible for the activation of the β-catenin signalling pathway in carcinoma.
Box 3 | What constitutes the tumour microenvironment?

The tumour microenvironment, in addition to harbouring carcinoma cells, consists of various components that have a major role in influencing the outcome of the malignancy. These can be widely classified into three main groups: cells of haematopoietic origin, cells of mesenchymal origin and non-cellular components. Tumours of different origins and different stages of progression will inevitably contain components in various proportions. Nonetheless, these three classes of stromal components represent the most abundant elements present in the microenvironment of solid tumours.

**Cells of haematopoietic origin.** This compartment consists of cells that arise in the bone marrow and can be subdivided into cells of the lymphoid lineage, consisting of T cells, B cells and natural killer (NK) cells, and those of the myeloid lineage, which includes macrophages, neutrophils and myeloid-derived suppressor cells (MDSCs). The roles of different subsets of T cells in tumour promotion and tumour elimination have been well studied and are the subject of recent advances in the development of novel immunotherapies. The alternative activation of macrophages and their ability to promote tumour development and progression have also been well documented. Similarly, each of the other constituent cell types has either a positive or negative effect on the outcome of the tumour. Importantly, interactions between different cell types within the stroma can also have a major role in tumour progression, as has been shown, for example, for CD4 T cells and macrophages. Other cell types that have a major role in tumorigenesis but are not abundantly present in the tumour microenvironment per se include platelets and dendritic cells.

**Cells of mesenchymal origin.** These comprise cells derived from the mesenchyme and include fibroblasts, myofibroblasts, mesenchymal stem cells (MSCs), adipocytes and endothelial cells. Fibroblasts and MSCs derived from the bone marrow have been shown to directly support cancer stem cell (CSCs) by creating a favourable niche and facilitating tumour progression. Until recently, adipocytes were thought of only as energy storage houses; however, recent studies have revealed the importance of factors secreted by adipocytes (for example, hepatocyte growth factor; HGF) in tumour progression. Endothelial cells and pericytes that constitute the walls of blood vessels have a major role in vascular functionality and angiogenesis, as well as the regulation of cancer cell dissemination.

**Non-cellular components.** The major non-cellular component of the tumour microenvironment is the extracellular matrix (ECM), which consists of many distinct components — including proteins, glycoproteins and proteoglycans — that enable its functions both structurally and functionally. The ECM can be subdivided into the more compact basement membrane — which is a specialized ECM that is rich in type IV collagen, laminin and fibronectin — and the interstitial matrix, which consists of fibrillar collagens, proteoglycans and glycoproteins that contribute to the tensile strength of the tissue. The ECM is involved in the formation of a stem cell niche and although it acts to maintain tissue architecture and prevent cancer cell invasion, an abnormal ECM has been shown to promote tumour progression and tumour angiogenesis.

Together, these components make up the tumour microenvironment, which represents a complex habitat involving myriad interactions between cell types and the ECM, each having a role in influencing tumour outcome. The figure depicts the tumour microenvironment, showing all the major constituents mentioned above.

Cells; once activated, this signalling promotes their acquisition of a CSC phenotype. Similar reciprocal interactions exist in breast cancers, in which MSCs recruited from the bone marrow interact with carcinoma cells via paracrine cytokine signalling involving CXC-chemokine ligand 7 (CXCL7; also known as platelet basic protein) and interleukin-6 (IL-6), which are both responsible for stimulating the self-renewal of neoplastic cells. Hence, MSCs secrete cytokines and growth factors that together create a suitable niche enabling carcinoma cells to acquire and maintain stemness. Similar roles have been reported for tumour-associated macrophages (TAMs), which secrete factors such as IL-6 that activate the JAK (Janus kinase)–STAT (signal transducer and activator of transcription) pathway within the tumour cells, thus enhancing their tumorigenicity and resistance to chemotherapy by imparting CSC properties to them.

In principle, the rapidly accumulating understanding of the paracrine signalling pathways that activate and sustain the CSC programme should provide insights for targeting CSCs; such a focused approach would represent an alternative to the untargeted use of high-throughput screening. For example, one means of blocking pathways activated by stroma-derived signals could involve using antagonists of the PGE receptor subtype EP4 (PTGER4), such as the small molecule RQ-15986, thereby reversing the tumour-promoting effects of MSCs on carcinoma cells. Similarly, inhibitors of the binding of STAT3 to DNA, such as the small molecule NSC 74859, may be effective in blocking the immune cell-activated IL-6–STAT3 signalling that supports CSC properties in certain carcinomas.

Of course, such approaches may also result in the inhibition of signalling cascades in normal cells, in which these signals have crucial roles. For example, signalling via the canonical WNT pathway is known to be essential for the regulation and homeostasis of intestinal stem cells, and the use of WNT inhibitors might therefore lead to a depletion of the normal resident stem cell population that is responsible for the continuous regeneration of the intestinal epithelium. However, the dependency of colorectal CSCs on β-catenin signalling might exceed that of the normal intestinal stem cells, yielding a favourable therapeutic index such that a low-dose treatment might still be able to deplete CSC activity without substantially affecting normal organ homeostasis. Indeed, several clinical trials using inhibitors of
WNT signalling are currently underway, including an ongoing Phase I study of LGK974, an oral inhibitor of the protein cysteine N-palmitoyltransferase Porcupine for the treatment of patients with advanced breast and pancreatic cancer (ClinicalTrials.gov identifier: NCT01351103). Porcupine is a membrane-bound O-acyltransferase that is responsible for the post-translational maturation of WNTs via palmitoylation.

An alternative approach to disrupting stromal signals might involve preventing the cellular sources of these signals from being recruited into the tumour stroma in the first place. For instance, MSCs are often recruited into the tumour-associated stroma by carcinoma cell-derived IL-8 (REF. 86). In such cases, inhibition of this homing signal might be effective in preventing the localization of MSCs to the tumour stroma and the resulting development of a supportive CSC niche. Similar tropic signals have also been reported for macrophages, which home to the primary tumour in response to factors such as macrophage colony-stimulating factor (M-CSF; also known as CSF1) and several chemokines, including CCL2, CCL5 and CXCL12 (REFS 87–89). Antibodies that block the M-CSF receptor (CSF1R; also known as FMS) have been developed and shown to be effective at reducing the numbers of TAMs homing to tumours in syngeneic mouse tumour models80. Similarly, inhibitors of the tyrosine kinase activity of CSF1R have been developed and shown to inhibit the tumour-promoting effects of macrophages, including the acquired resistance of tumour cells to chemotherapy81. A CSF1R kinase inhibitor, the small molecule ARRY-382, has recently gone through Phase I clinical trials in patients with metastatic cancers (ClinicalTrials.gov identifier: NCT01316822) and may be the forerunner of a large repertoire of agents that can be used either individually or in combination with other drugs to prevent macrophage homing.

Inhibition of CSC-dependent pathways. One strategy for blocking the initiation of the EMT programme, as well as entrance into and maintenance of the CSC state, has already been suggested by findings cited above. Thus, epithelial non-CSCs synthesize and secrete high levels of physiological inhibitors of WNT signalling, such as SFRP and DKK proteins, which act at the cell surface to block ligand binding–mediated activation of Frizzled receptor signalling82. As argued above, these secreted inhibitors ostensibly serve to reinforce residence in the epithelial state. Similarly, secreted inhibitors of the TGFβ pathway, including Gremlin and the BMPs (BMP4, BMP6, BMP7, BMP9 and BMP10), are expressed and secreted at high levels in non-CSCs, where they appear to block another pathway (involving TGFβ) that is crucial for the maintenance of the non-CSC (epithelial) state83. Derivatives of these secreted inhibitory molecules, acting in the extracellular space, could become potential therapeutics that prevent stochastic interconversion between the CSC and non-CSC states, tilting the balance in favour of the non-CSC state. However, therapeutic proteins such as these are difficult and expensive to produce in large quantities and often difficult to deliver into the interstices of complex tissues such as tumours.

Given the importance of WNT signalling in the induction of the CSC state, several low-molecular-weight inhibitors of this pathway have been developed. One of the first inhibitors to target the WNT pathway was ICG-001. ICG-001 is a small molecule that blocks the ability of CREB-binding protein (CBP), a co-factor for several transcription factors, to act as a co-activator of the β-catenin–TCF (T cell factor) complex, which is the transcription factor complex activated by canonical WNT signalling84. As mentioned above, inhibitors of the WNT pathway that target the Porcupine enzyme — the small molecules IWP2 (REF. 94) and LGK974 (REF. 95) — have also been developed. These inhibitors may be effective in preventing the secretion of active WNT molecules by stromal cells, thereby blocking the paracrine signalling that triggers the formation of new CSCs. In addition, they may block the autocrine signalling that is used by existing CSCs to maintain their residence in the stem cell state. Recent studies have also identified a role for yet another β-catenin co-factor, YAP1 (also known as YAP65), which forms a complex with β-catenin and is essential for the formation of β-catenin-driven cancers85. Reviews detailing the targeting of various aspects of the WNT86 and TGFβ87 pathways have been published elsewhere.

Several molecules targeting various nodes of the Hedgehog and Notch pathways are also coming into prominence for their ability to target CSCs. For example, IPI926, a derivative of the natural product Smoothened (SMO) antagonist cyclopamine88, is undergoing clinical trials for malignancies such as basal cell carcinomas and metastatic pancreatic cancer in combination with chemotherapeutic drugs such as gemcitabine (ClinicalTrials.gov identifier: NCT01130142). Similar trials have also

**Figure 3 | Impact of stromal cells and secreted factors on cancer stem cells.** Each stromal cell type depicted here influences the tumour by secreting factors that stimulate the formation of cancer stem cells (CSCs) and help maintain the residence of already formed CSCs in the stem cell state. Summarized here are some of the major factors secreted by each cell type that are known to have an impact on CSCs. CXCL7, CXC-chemokine ligand 7; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IL-6, interleukin-6; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; OncoM, oncostatin M; PDGF, platelet-derived growth factor; PGE2, prostaglandin E2; SDF1, stromal cell-derived factor 1; TGFβ, transforming growth factor-β.
Table 2 | Potential pathways for specific targeting of CSCs and potential drug candidates

| Pathway | Targets | Compounds | Potential indications | Clinical development |
|---------|---------|-----------|----------------------|----------------------|
| WNT     | Porcupine | IWP2 (REF. 94), LGK974 (REF. 95), | Breast cancer: maintenance of breast CSCs through recruitment of WNT ligands\(^{216}\) | LGK974 is in Phase I trial for malignancies dependent on WNT ligands, including melanoma, breast and pancreatic malignancies (ClinicalTrials.gov identifier: NCT01351103) |
| β-catenin | | ICG-001 (REF. 93), PRI-724, OXT-328 (REF. 219), iCRT\(^{11}\), CWP232291 (REF. 221) | Colon cancer: required for maintenance of colon CSCs through signals from stroma\(^{36}\) | CWP232291 (REF. 221), which induces β-catenin degradation, is in Phase I trials for AML and CML (ClinicalTrials.gov identifier: NCT01398462) |
| | | | AML and CML: could prove to be effective in MLL-fusion-driven CSCs in AML, and in imatinib-resistant CSCs in CML\(^{22,23}\) | PRI-724, an inhibitor of the β-catenin-CBP interaction, is in Phase I trials for advanced solid tumours (ClinicalTrials.gov identifier: NCT01302405) |
| AXIN2 (tankyrase inhibition) | | XAV-939 (REF. 220), IWR\(^{34}\) | Breast cancer: degradation of β-catenin blocks the generation of breast CSCs\(^{219}\) | |

| TGFβ | TGFβR1 | LY2157299 (REF. 225) | Breast cancer: increased TGFβ signalling in CSCs of chemotherapy-resistant breast cancer\(^{216}\) | LY2157299 is in Phase I trials for recurrent malignant glioma (ClinicalTrials.gov identifier: NCT01667187) |
| TGFβ | TGFβR2 | GC1008 (REF. 228); also known as fresolimumab, AP12009 (REF. 229) | Colon cancer: increased TGFβ signalling in CSCs of chemotherapy-resistant breast cancer\(^{216}\) | |
| Notch | γ-secretase | MK-0752 (REF. 230), PF03084014 (REF. 231), R04929097 (REF. 252) | Breast cancer: Notch signalling regulates breast CSCs\(^{213}\) | MK-0752 is in a Phase I trial for advanced breast cancer (ClinicalTrials.gov identifier: NCT00106145) |
| | | | Ovarian cancer: Notch signalling is critical for the regulation of CSCs and platinum resistance\(^{11}\) | PF-03084014 is in Phase I trials for metastatic breast cancer (ClinicalTrials.gov identifier: NCT01876251) |
| | | | | R04929097 is in a Phase I trial for advanced solid tumours\(^{11}\) (ClinicalTrials.gov identifier: NCT01145456) |
| | | | | AP12009 is in a Phase I trial for melanoma and pancreatic and colorectal neoplasms (ClinicalTrials.gov identifier: NCT00844060) |
| DLL4 | | MEDI0639 (REFS 105,237) | Colon cancer: blocking DLL4 reduces tumour-initiating frequency\(^{315}\) | MEDI0639, a DLL4-specific antibody, is in a Phase I trial for advanced solid tumours (ClinicalTrials.gov identifier: NCT01577745) |
| Hedgehog (Hh) | SMO | Cyclopamine\(^{258}\), GDC-0449 (REF. 239), LDE225 (REF. 240) | Breast cancer: Hh signalling regulates self-renewal of breast CSCs\(^{216}\) | LDE225 is in a Phase I trial for advanced solid tumours (ClinicalTrials.gov identifier: NCT01576666) |
| | | | | A combination of Notch and SMO inhibitors (RO04929097 and GDC-0449) is in a Phase I trial for metastatic breast cancer (ClinicalTrials.gov identifier: NCT01071564) |
| JAK–STAT | IL-6 | CNT0328 (REF. 246), Tocilizumab (REF. 247) | Breast cancer: IL-6 is required for the multipotency of CSCs\(^{248}\) | CTN0328 is in a Phase II trial for advanced prostate cancer (ClinicalTrials.gov identifier: NCT00433446) |
| | JAK1, JAK2 | AZD1480 (REF. 250), WP1066 (REF. 255); also inhibits STAT3 | Breast cancer: JAK1/STAT3 pathway inhibits CSC function\(^{311}\) | WP1066 is in a Phase I trial for glioblastoma and other solid tumours (ClinicalTrials.gov identifier: NCT01904123) |
| | | | | OPB-31121 is in a Phase I trial for advanced solid tumours (ClinicalTrials.gov identifier: NCT00955812) |
| STAT3 | | Compound 1 (REF. 252), OPB-31121 (REF. 253) | Breast cancer: the JAK–STAT pathway is required for breast CSC signalling\(^{315}\) | |
| PDGFR | PDGFR, PKCa | Bisindolylmaleimide I (REF. 254), ISIS 3521 (REF. 255) | Breast cancer: PDGFR signalling is required for the survival of breast CSCs\(^{14}\) | ISIS 3521 is in a Phase II trial for metastatic breast cancer (ClinicalTrials.gov identifier: NCT00003236) |

AML, acute myeloid leukaemia; AXIN2, axis inhibition protein 2; CBP, CREB-binding protein; CML, chronic myeloid leukaemia; CSC, cancer stem cell; DLL4, Delta-like protein 4; EMT, epithelial-to-mesenchymal transition; IL-6, interleukin-6; JAK, Janus kinase; LSC, leukaemic stem cell; MLL, mixed lineage leukaemia; PDGFR, platelet-derived growth factor receptor; PKCa, protein kinase Ca; SMO, Smoothened; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor-β; TCF, T cell factor; TGFβR1, TGFβ receptor 1.

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been shown to induce the differentiation of suberoylanilide hydroxamic acid (SAHA), vitamin D, and antibodies targeting Delta-like protein 4 of Notch are promising candidates for reducing the CSC frequency of breast tumour xenographs and other solid tumours. A list of inhibitors targeting pathways that are preferentially utilized by CSCs and that may possess therapeutic utility is summarized in Table 2. However, these studies do not clarify the therapeutic indices that these agents will exhibit: that is, will they in fact target neoplastic CSCs far more effectively than the stem cells residing in corresponding normal tissues?

**Differentiation therapy to curb stem-like properties of CSCs.** One of the first successes of a targeted therapy for any type of cancer involved the use of all-trans retinoic acid (ATRA), which was given to patients suffering from PML–RARα-induced acute promyelocytic leukaemia. Following treatment with ATRA, leukaemic promyelocytes are relieved of their blockade of differentiation and able to differentiate into mature granulocytes. The success of this therapy has led to the theory that differentiation therapy may be effectively used to treat other forms of cancer. In the case of CSCs, notably those in carcinomas, the notion would be to induce their exit from the CSC state into the more differentiated epithelial state of non-CSCs.

Other agents have similarly been shown to be beneficial in differentiation therapy. These include phorbol myristate acetate (PMA), which is capable of inducing leukaemic cell differentiation by, among other pathways, protein kinase C (PKC)-mediated induction of transcription factors such as PU.1 (also known as SPI1) and AP1, which induces the differentiation of human leukaemia cells through several distinct mechanisms; dimethylsulfoxide (DMSO), which induces the differentiation of human promyelocytic leukaemias; and vitamin D, which induces the maturation of several leukaemic cell lines.

However, other compounds such as suberoylanilide hydroxamic acid (SAHA), a histone deacetylase (HDAC) inhibitor, have been shown to induce the differentiation of human breast cancer cells and endometrial carcinomas. Inhibition of HDACs is thought to release the repression of gene promoters that have major roles in the processes of differentiation and cell cycle arrest, thereby permitting their expression. Another functional class of compounds that induce cell differentiation is represented by 5-azacytidine, a chemical analogue of cytidine, which acts by inactivating the DNA methyltransferases that catalyse the methylation of CpG islands and associated transcriptional repression at gene promoters.

Growing interest in the field of epigenetics research (defined here as the study of various types of chromatin modifications) is uncovering a large number of histone methyltransferases and histone demethylases. Modulation of these enzymes might also result in increased transcription of genes that are important for CSC differentiation or silence those that are required for cell migration and invasion. For example, the promoter of the CDH1 gene, which encodes E-cadherin, the keynote of the epithelial state, is a target of silencing in many cancers through the actions of the Polycomb repressive complex 2 (PRC2), which imposes the K27me3 repressive mark on histone H3. However, inhibitors of histone-modifying enzymes, such as the methyltransferase EZH2, could act as differentiation-inducing agents of CSCs that have undergone an EMT by inducing the re-expression of E-cadherin and thereby restoring epithelial properties. In fact, the small-molecule EZH2 inhibitor E7438 is currently in Phase I/II clinical trials for advanced solid tumours and B cell lymphomas (ClinicalTrials.gov identifier: NCT01897571). A more detailed review of the epigenetic regulation of the EMT and CSCs has recently been published.

All differentiation-inducing agents act on the premise that a blockade of terminal differentiation is one of the major characteristics of the neoplastic state. The realization of this phenomenon came from studying leukemias and analysing the phenotypes of cancer cells in relation to the normal differentiation hierarchy of the haematopoietic system. Thus, acute myeloid leukaemia caused by the MLL–AF9 chromosomal translocation leads to the expansion of leukaemic stem cell populations that exhibit a cell-surface antigen profile similar to that of granulocyte–monocyte progenitors. In the case of MLL–AF9-driven leukemias, it is known that cells are maintained in the less differentiated progenitor stage by the fusion protein, which activates target genes such as homeobox A9 (HOXA9) and MEIS1 that are responsible for coordinating a downstream CSC programme through aberrant methylation of H3K79 by the histone methyltransferase DOT1L.

Hence, inhibitors of DOT1L may specifically act to disrupt the MLL–AF9-induced transcriptional programme that fuels these cancers.

A clinically effective differentiation therapy would need to be combined with chemotherapy in order to eradicare both CSC and non-CSC populations within a tumour. Recent studies have focused on such combinations; these involve inhibition of TGFβ, which results in the differentiation of triple-negative breast CSCs, in combination with paclitaxel, which should eradicate the rapidly cycling non-CSCs forming the bulk of tumours under treatment. Targeted therapy would presumably act by depleting CSC potential, giving rise to non-CSC counterparts that are more susceptible to chemotherapy. In fact, the use of agents that specifically target CSGs in combination with conventional chemotherapy has already been evaluated in clinical trials that have been designed to gauge the efficacy of the targeted agents in combination with radiotherapy for the treatment of metastatic breast cancers (ClinicalTrials.gov identifier: NCT01401062).

One proviso must be mentioned here: it has been suggested that disseminated carcinoma cells must be able to undergo a mesenchymal-to-epithelial transition (MET) at their site of dissemination in order to generate the mixed CSC plus non-CSC populations that seem to be crucial for the robust growth of a tumour. Hence, although it will still inhibit CSC formation and maintenance in the primary tumour, inducing CSC differentiation by inducing a MET may inadvertently support the process of metastatic colonization at distant sites. The complex dynamics of these reversible processes operating during the invasion-to-metastasis cascade remain to be elucidated.

**Directed immunotherapy against CSCs.** The past decade has seen a resurgence of the idea that the immune system can be directed against tumour-specific and tumour-associated antigens in patients with cancer. Strategies that exploit our understanding of the immune system for cancer therapy include the use of specific peptides that are derived from tumour antigens, which could be used for cancer vaccination as immunotherapy, the use of dendritic cells as vaccines to activate adaptive immune cells against specific antigens, and the blockade...
of immune checkpoints that inhibit antitumour immune responses\textsuperscript{128}. T cell responses to a particular antigen are dictated by the nature of their interaction with an antigen-presenting cell, and depend on co-stimulatory and inhibitory signals that are delivered following the binding of specific ligands from antigen-presenting cells to complementary receptors on T cells. The modulation of these so-called immune checkpoints by antigens of inhibitory signals has been recognized as a major avenue for the development of novel immunotherapies\textsuperscript{124}. Drugs such as ipilimumab, a cytotoxic T lymphocyte antibody, have been found to confer a clear benefit in treating melanoma\textsuperscript{125,126}, with clinical trials underway for several other malignancies, including prostate cancer (ClinicalTrials.gov identifier: NCT01194271) and ovarian cancer (ClinicalTrials.gov identifier: NCT01611558).

Accordingly, the exploitation of the immune system may yield another dimension of anti-CSC therapy by directing immunocytes to recognize CSCs through the unique collection of cell-surface antigens displayed by these cells. However, another process may deflect immune attacks on tumours: the autocrine secretion of TGF\(\beta\) (a known immunosuppressant) by CSCs may create zones around these cells that are protected from attack by various cellular components of the immune system\textsuperscript{90,127}. In addition, CSC-secreted TGF\(\beta\) may cause the formation of regulatory T cells (Treg cells) that have additional immunosuppressive effects on other subclasses of T lymphocytes\textsuperscript{128}.

As described earlier, the identification of cell-surface markers on CSCs (TABLE 1) has enabled the isolation of these cells from primary tumours. Such markers could be used to survey tumour antigens that are preferentially expressed on the CSCs compared to the bulk of tumour cells and reveal novel vulnerabilities that could be exploited to design stem cell-specific immunotherapies. Additionally, the expression levels of certain potentially inhibitory receptors that could attenuate an immune response could be higher in CSCs than in non-CSCs; when these inhibitory receptors are blocked, it may be possible to achieve a more uniform and long-term immune response against both CSC and non-CSC populations.

**Exploiting metabolic differences to target CSCs.** The field of cancer metabolism has undergone a revival over the past decade with renewed interest in the Warburg effect, which proposes that cancer cells generate ATP through glycolysis rather than oxidative phosphorylation, even under non-hypoxic conditions\textsuperscript{129}. Through several key breakthrough studies, the role of cell metabolism has evolved into an active area of cancer research with the development of novel therapeutics based on our understanding of the signals that regulate metabolic pathways\textsuperscript{130}; these include tamsorilimus (Torisel; Wyeth/Pfizer) and everolimus (Afinitor; Novartis), inhibitors of mammalian target of rapamycin complex 1 (mTORC1), which are approved for the treatment of several cancers\textsuperscript{131–138}.

Hypoxia within a tumour can lead to alterations in the metabolism of cancer cells through the inhibition of mTOR signalling\textsuperscript{139}. The hypoxic conditions that are apparent in many — if not most — tumours have also been shown to lead to the induction of an EMT and the generation of CSCs\textsuperscript{90,141}. This leads to the speculation that hypoxia-induced metabolic changes could contribute to the mesenchymal or CSC phenotype. Indeed, a recent study reported that a metabolic switch to glycolysis occurs upon activation of the EMT-inducing transcription factor SNAIL, and that blockade of this switch through ectopic expression of fructose-1,6-biphosphatase abrogates the ability of SNAIL to impart mesenchymal or stem-like properties to breast cancer cells\textsuperscript{142}. Other studies also indicate that components of the mevalonate metabolic pathway are important in the generation of breast CSCs, with inhibition of the pathway using hydroxy-3-methylglutaryl CoA reductase blockers resulting in a reduction in CSC properties\textsuperscript{143}. These studies suggest that alteration of the metabolic phenotype could be an essential step that is required for entrance into the CSC state, and that studying the differences in metabolism between CSCs and non-CSCs may reveal novel ways by which we can specifically target these cells.

**Conclusions**

The avenues discussed above represent only some of the possible ways by which CSCs could be targeted therapeutically. For example, another strategy that has not been discussed here involves the delivery of short hairpin or small interfering RNAs that can induce a CSC-specific knockdown of crucial genes identified through large-scale screening. Synthetic lethality studies might also uncover novel dependencies of CSCs that could be candidates for future targeting studies.

There are, however, multiple hurdles that have to be surmounted before we can effectively eliminate these cells. First, it is apparent that CSCs exist in a specific niche formed by multiple cell types and distinct signalling molecules, including growth factors, cytokines and extracellular matrix components. Hence, studying the biological properties of CSCs in isolation or performing screens with them in the absence of a surrounding relevant niche is unlikely to yield results that can be translated to responses occurring \textit{in vivo}. Second, owing to the lack of appropriate experimental systems to accurately model human tumours, at least at present, we are forced either to study human tumours in immunodeficient mice in the absence of an adaptive immune system or to study mouse tumours in a syngeneic setting. Neither of these experimental settings accurately recapitulates the biological complexity of the tumours encountered in the oncology clinic.

The current practice of modelling human tumours by the alteration of one or two genetic loci in the cells of mice cannot provide a fair representation of the intricate nature of the disease and may not lead to the development of therapies that translate directly to humans. Moreover, mouse and human cells have fundamental differences in their intracellular wiring, which lead to differences in the processes underlying tumorigenesis in these two species\textsuperscript{144}. Hence, we need to devise novel and appropriate strategies for studying primary human tumour xenografts in ways that preserve their CSC subpopulations in physiological states similar to those that existed prior to their isolation from patients. The use of mice that harbour a human immune system (humanized mice)\textsuperscript{145} may partly compensate for the lack of an adaptive immune system; for example, the macrophages that home to tumours growing as xenografts in such mice would be of human origin, and would secrete factors that may serve to model a CSC niche more similar to the one found in the human body. Ultimately, using better models we should be able to develop a more detailed and nuanced understanding of the essential nature of CSCs and the mechanisms supporting them within human tumours.

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Competing interests statement
The authors declare no competing interests.

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