TUMOUR STEM CELLS AND DRUG RESISTANCE

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Abstract | The contribution of tumorigenic stem cells to haematopoietic cancers has been established for some time, and cells possessing stem-cell properties have been described in several solid tumours. Although chemotherapy kills most cells in a tumour, it is believed to leave tumour stem cells behind, which might be an important mechanism of resistance. For example, the ATP-binding cassette (ABC) drug transporters have been shown to protect cancer stem cells from chemotherapeutic agents. Gaining a better insight into the mechanisms of stem-cell resistance to chemotherapy might therefore lead to new therapeutic targets and better anticancer strategies.

The discovery of cancer stem cells in solid tumours has changed our view of carcinogenesis and chemotherapy. One of the unique features of the bone-marrow stem cells that are required for normal haematopoiesis is their capacity for self-renewal. In the haematopoietic system, there are three different populations of multipotent progenitors — stem cells with a capacity for long-term renewal, stem cells with a capacity for short-term renewal, and multipotent progenitors that cannot renew but differentiate into the varied lineages in the bone marrow[1–3]. The multipotent progenitors and their derived lineages undergo rapid cell division, allowing them to populate the marrow. The factors that determine the self-renewing capacity of a cell, and how cancer cells acquire this ability, are not yet understood.

Pluripotent stem cells that possess both self-renewal capabilities and the ability to generate an organ-specific, differentiated repertoire of cells exist in organs other than the haematopoietic system and these can be studied to gain better insight into the stem-cell biology of a tumour. The concept of organ stem cells is difficult when one considers the many different cell types and functions of an organ, but emerging evidence indicates such pluripotent stem cells exist. In the normal mammary gland, for example, three cell lineages have been described — myoepithelial cells that form a basal cell layer, ductal epithelial cells, and milk-producing alveolar cells[4]. Although transplantation studies in mice have demonstrated that most mammary cells have a limited capacity for self-renewal, clonal populations that can recapitulate the entire functional repertoire of the gland have been identified[5]. In an elegant study, human mammary epithelial cells derived from reduction mammoplasties were used to generate non-adherent spheroids (designated mammospheres) in cell culture and demonstrate the presence of the three mammary cell lineages. More importantly, the cells in the mammospheres were clonally derived, providing evidence for a single pluripotent stem cell[4]. These same approaches are being used to isolate and characterize breast cancer stem cells.

In the haematopoietic system as well as in other normal tissues, the normal stem cell must be both self-renewing and pluripotent. Although stem cells can self-renew, they are generally quiescent, spending most of their time in G0. Because stem cells can repair their DNA as they self-renew, they have the potential to accumulate mutations acquired after exposure to carcinogens. If tumours arise from stem cells, the accumulation of these mutations might be what we have come to recognize as the 'multistep process of carcinogenesis'. So do cancer stem cells arise from normal stem cells, or do they arise from differentiated cells that acquire self-renewal capacity, or both? Does the innate resistance of normal stem cells to radiation and toxins contribute to the failure of some cancer

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TERATOCARCINOMAS
Malignant germ-cell tumours that exhibit cell phenotypes that are derived from more than one of the three primary germ-cell layers (endoderm, mesoderm, ectoderm).

Summary

- Stem-cell populations have been identified in a range of haematopoietic and solid tumours, and might represent the cell of origin of these tumours.
- Normal and cancer stem cells express high levels of ATP-binding cassette (ABC) transporters, such as *ABCB1*, which encodes P-glycoprotein, and the half-transporter *ABCG2*, which was originally identified in mitoxantrone-resistant cells.
- The drug-transporting property of stem cells conferred by ABC transporters is the basis for the 'side-population' phenotype that arises from the exclusion of the fluorescent dye Hoechst 33342.
- Cancer stem cells are likely to share many of the properties of normal stem cells that provide for a long lifespan, including relative quiescence, resistance to drugs and toxins through the expression of several ABC transporters, an active DNA-repair capacity and a resistance to apoptosis. Therefore, tumours might have a built-in population of drug-resistant pluripotent cells that can survive chemotherapy and repopulate the tumour.

therapies? How can we exploit our knowledge of stem-cell biology to specifically target these cells and improve therapy?

Cancer stem cells

Cells with stem-cell qualities have been identified in malignancies of haematopoietic origin and in some solid tumours. The existence of such a population would imply that the stem cell represents the cell of origin for the tumour, as illustrated in FIG. 1. One can predict that such cancer stem cells represent only a small fraction of a tumour, as they possess the capability to regenerate a tumour, and most cancer cells lack this regenerative capability. For example, when plated in soft agar or injected into mice, most tumour cells do not give rise to colonies[6,7]. Similarly, in experiments performed in humans in the 1950s, unthinkable by today's ethical standards, 35 patients had an estimated one billion of their own tumour cells injected into their thigh or forearm[8].

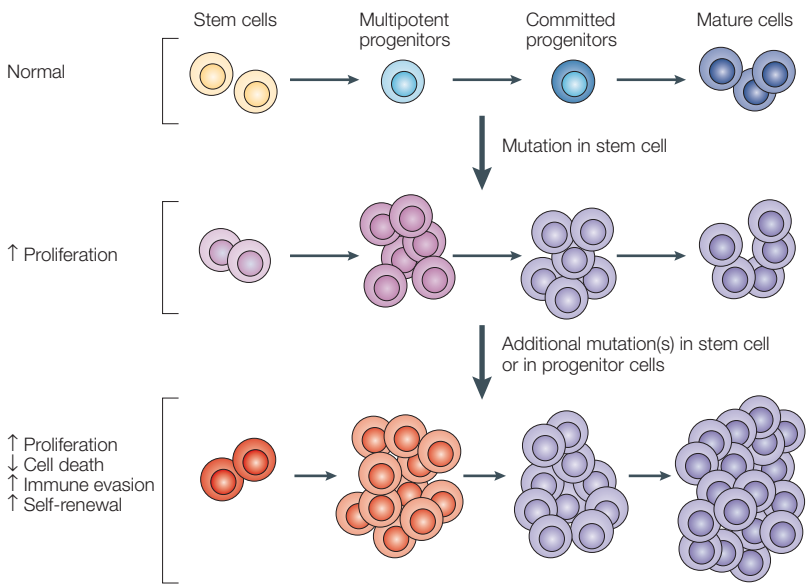


Figure 1 | **Cancer stem cells and tumour progression.** Normal stem cells give rise to multipotent progenitor cells, committed progenitors and mature, differentiated cells. Mutations in a stem cell give rise to a stem cell with aberrant proliferation and result in a pre-malignant lesion. Additional mutations lead to the acquisition of further increased proliferation, decreased apoptosis, evasion of the immune system, and further expansion of the stem-cell compartment that is typical of malignant tumours.

'Only' 7 of these autotransplants resulted in tumour growth at the injection site. Furthermore, studies of acute myelogenous leukaemia have shown that only 0.1–1% of all cells have leukaemia-initiating activity[9]. These leukaemia-initiating cells have many markers and properties of normal haematopoietic stem cells[10,11]. So it is believed that leukaemia arises from a stem cell that becomes transformed and gives rise to a large population of clones that proliferate but cannot self-renew or fully differentiate[12]. Similar populations of self-renewing cells, such as those that carry the chromosomal translocation t(9;22)(q34;q11), which forms the *BCR–ABL* fusion gene, have also been identified in patients with chronic lymphocytic leukaemia and chronic myelogenous leukaemia (CML)[13].

Evidence for the existence of a pluripotent cell in solid tumours includes clinical observations with human TERATOCARCINOMAS, an experiment of nature in which differentiated tissues such as muscle and bone can appear in the tumour mass[14–16], and from the observation that mouse teratocarcinoma cells can produce a normal mouse[17]. Instead of haematopoietic markers, stem cells identified from solid tumours usually express organ-specific markers. In eight of nine human breast cancer samples, for example, a tumorigenic stem-cell population was found that expressed the unique cell-surface marker profile $CD44^{+}CD24^{-/low}Lin^{-}$ (REFS 4,18). This population was enriched 50- to 100-fold with cells able to form tumours in mice. The resulting tumours possess the phenotypic heterogeneity found in the original tumour population, including both tumorigenic and non-tumorigenic cells. In another study, overexpression of the WNT family of genes, important regulators of normal cell development, led to expansion of the mammary-stem-cell pool and cancer susceptibility[19]. Finally, stem cells with a capacity to self-renew and undergo pluripotential differentiation have been isolated from human central-nervous-system tumours[20–22]. These cells were reported to express CD133 — a cell-surface antigen known originally as a marker of haematopoietic stem cells and later observed as a marker of stem cells in other normal tissues[23–26].

The exact origin of pluripotent stem cells in tumours might vary. They could arise from the malignant transformation of a normal stem cell that has accumulated oncogenic insults over time. Alternatively, the original

Table 1 | **ABC transporters involved in drug resistance**

Gene	Protein/alias	Chemotherapeutic drugs effluxed by transporter	Other drugs and substrates
ABCA2	ABCA2	Estramustine	–
ABCB1	PGP/MDR1	Colchicine, doxorubicin, etoposide, vinblastine, paclitaxel	Digoxin, saquinivir,
ABCC1	MRP1	Doxorubicin, daunorubicin, vincristine, etoposide, colchicine, camptothecins, methotrexate	Rhodamine
ABCC2	MRP2	Vinblastine, cisplatin, doxorubicin, methotrexate	Sulfinpyrazone
ABCC3	MRP3	Methotrexate, etoposide	–
ABCC4	MRP4	6-mercaptopurine, 6-thioguanine and metabolites; methotrexate	PMEA, cAMP, cGMP
ABCC5	MRP5	6-mercaptopurine, 6-thioguanine and metabolites	PMEA, cAMP, cGMP
ABCC6	MRP6	Etoposide	–
ABCC11	MRP8	5-fluorouracil	PMEA, cAMP, cGMP
ABCG2	MXR/BCRP	Mitoxantrone, topotecan, doxorubicin, daunorubicin, irinotecan, imatinib, methotrexate	Pheophorbide A, Hoechst 33342, rhodamine

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanine monophosphate; MDR, multidrug resistance; MRP, multidrug-resistance-associated protein; MXR, mitoxantrone resistance protein; PMEA, 9-[2-(phosphonomethoxy)ethyl]adenine.

tumour cell could be a more differentiated cell that develops the capacity for continual self-renewal, thus acquiring the properties of a stem cell[27]. Distinguishing between these two might be difficult. Evidence that cells other than stem cells can acquire the ability to undergo self-renewal has been recently provided in studies examining the progression of CML[13]. The chronic phase of the disease occurs when a stem cell acquires the expression of the BCR–ABL fusion protein, leading to increased proliferation of cells within the granulocyte–macrophage progenitor pool and their downstream progeny. It is hypothesized that progression to BLAST CRISIS follows additional genetic or epigenetic events that confer progenitor cells with the capacity to self-renew, making them indistinguishable from a leukaemic stem cell. Further proof is needed to confirm that progression to blast crisis occurs at the level of the progenitor pool, but the proposal that the stem-cell compartment is not rigidly defined is attractive and suggests a degree of plasticity in cancer.

Cancer stem cells (with either inherent or acquired capabilities for self-renewal) give rise to cells that lack long-term self-renewal capability but retain a finite ability to divide. In normal physiology, this would be called 'differentiation', as the cell acquires traits specific to its place in the tissue. But in cancer, cells lack the ability to undergo differentiation into phenotypically mature cells. A limited amount of differentiation often does occur, giving rise to the well-known histopathological and molecular distinctions between tumours. In fact, the further along this pathway the cancer cell travels, the more differentiated and the more like its normal counterpart it becomes, accordingly demonstrating a slower growth rate. Where the so-called 'de-differentiated' tumours fit along this continuum is uncertain, but it is possible that self-renewal might be a property that represents a higher order of differentiation.

Therefore, the cancer stem cell shares many properties of the normal stem cell. It is generally accepted that normal stem cells show properties that provide for a long lifespan such as relative quiescence, resistance to drugs and toxins through the expression of several ATP-binding cassette (ABC) transporters, an active DNA-repair capacity, and a resistance to apoptosis. It follows that cancer stem cells might also possess these resistance mechanisms. The paradigm that drug resistance originates in the stem-cell phenotype might stimulate new strategies for the development of anticancer therapies.

Drug transporters in stem cells

Stem cells have many properties that separate them from mature, differentiated cells. In addition to their ability to self-renew and differentiate, they are quiescent, dividing infrequently. They also require specific environments comprising other cells, stroma and growth factors for their survival[28]. One particularly intriguing property of stem cells is that they express high levels of specific ABC drug transporters. For example, haematopoietic stem cells express high levels of *ABCG2*, but the gene is turned off in most committed progenitor and mature blood cells[29]. The two ABC-transporter-encoding genes that have been studied most extensively in stem cells are *ABCB1*, which encodes P-glycoprotein[30], and *ABCG2* (REFS 29,31–34). Along with *ABCC1*, they represent the three principal MULTIDRUG-RESISTANCE genes that have been identified in tumour cells. These genes, members of the ABC-transporter superfamily, are promiscuous transporters of both hydrophobic and hydrophilic compounds[30,35] (TABLE 1). These transporters also have important roles in normal physiology in the transport of drugs across the placenta and the intestine (more accurately, the retention of drugs in the intestinal lumen), and are important components of the blood–brain and blood–testis barriers. By using the energy of ATP hydrolysis, these transporters actively efflux drugs from cells, serving to protect them from cytotoxic agents[35–37]. Mice deficient in either *Abcg2*, *Abcb1* or *Abcc1* are viable, fertile and have normal stem-cell compartments[36,38,39]. This indicates that none of these

BLAST CRISIS
In patients with chronic myelogenous leukaemia, this term describes the progression of the disease to an acute advanced phase, evidenced by an increased number of immature white blood cells in the circulating blood.

MULTIDRUG RESISTANCE
Simultaneous resistance to several structurally unrelated drugs that do not necessarily have a common mechanism of action.

genes are required for stem-cell growth or maintenance. However, these knockout mice are more sensitive to the effects of drugs such as vinblastine, ivermectin, topotecan and mitoxantrone, consistent with a role for these ABC transporters in protecting cells from toxins.

The drug-transporting property of stem cells conferred by these ABC transporters is an important marker in the isolation and analysis of haematopoietic stem cells. Most cells accumulate the fluorescent dyes Hoechst 33342 and rhodamine 123, but stem cells do not, as these compounds are effluxed by *ABCG2* and *ABCB1*, respectively. Because they don't accumulate these fluorescent dyes, stem cells can be sorted by collecting cells that contain only a low level of Hoechst 33342 fluorescence. These cells are referred to as 'dull cells' or 'side population' (SP) cells. The term side population was coined because during flow-cytometry analysis, SP cells are visualized as a negatively stained 'side population' to one side of the majority of cells on a density dot plot. A large fraction of haematopoietic stem cells are found in the SP fraction[40] and when isolated from mice and transplanted into irradiated mice, small numbers of these SP cells can reconstitute the bone marrow, demonstrating that these cells are pluripotent. SP cells can be isolated from many tissues including the brain, breast, lung, heart, pancreas, testes, skin and liver, and these cells might represent lineage-specific stem cells[40–48]. Hoechst-33342 staining of bone marrow from ABCG2-null mice fails to detect SP cells. However, the lack of staining for SP cells occurs not because these cells are absent, but because the lack of ABCG2 expression allows these cells to accumulate Hoechst dye and become fluorescent.

SP cells in tumours and cell lines

Once it was recognized that stem cells were predominantly found in the SP fraction, it became possible to sort and purify stem cells from virtually any population of cells or tissue. SP cells were identified in 15 of 23 neuroblastoma samples and in neuroblastoma, breast cancer, lung cancer and glioblastoma cell lines[49]. Furthermore, analysis of several cell lines that had been maintained in culture for long periods of time demonstrated a small population of SP cells. In the rat glioma C6 cell line, a population of SP cells was seperated from a population of non-SP cells. Through the use of growth factors, investigators maintained these cells in culture, and showed that only the SP cells gave rise to both populations and produced cells with both neuronal and glial markers that were tumorigenic in mice[50]. This latter study provided strong evidence that in this cell line the SP population reflected a population with a capacity for self-renewal and limited maturation. However, this isolation approach is imperfect as the SP compartment is composed of stem and non-stem cells, and some stem cells are not in the SP fraction[38]. For example, non-stem-cell tumour cells often express *ABCG2* and *ABCB1*. These genes are highly expressed in drug-resistant cells, and histopathological studies have reported increased expression of the ABCB1 transporter in more differentiated tumours[51,52]. In addition, in a range of cell lines, differentiating agents induce expression of ABCB1, inhibit cell growth, and increase the expression of markers of maturation[53,54].

Additional limitations exist in using cancer cell lines cultured *in vitro* to study stem-cell biology and drug resistance. Although SP cells and cells with stem-cell properties have been reported in cultured cell lines, it is difficult to reconcile the hypothesis that only a small fraction of cells in culture possess stem-cell characteristics with the rapid doubling time of cells in culture. Current paradigms envision a small stem-cell compartment possessing cells with the capacity for perpetual self-renewal existing alongside a much larger proliferative compartment with cells that have a finite ability to proliferate before presumably arresting and/or undergoing apoptosis. These paradigms can explain the low cloning efficiency of most cell lines, their inefficiency at colony formation in soft agar, and their limited tumorigenicity. However, none of these models can explain how the stem cells remain a constant fraction of the total population, if indeed they do. Any proposal will require stem cells to divide slowly, and must recognize that in a cell line derived from a solid tumour the number of cells undergoing apoptosis is relatively small. One possibility is that there is an interchange of cells between a proliferative compartment and the stem-cell pool. That such an interchange might occur is not improbable, as the cell line almost certainly originated from a stem cell with a proliferative advantage. As discussed above, plasticity has already been proposed in CML.

Drug resistance in cancer cells

Cancer cells can acquire resistance to chemotherapy by a range of mechanisms, including the mutation or overexpression of the drug target, inactivation of the drug, or elimination of the drug from the cell. Typically, tumours that recur after an initial response to chemotherapy are resistant to multiple drugs (they are multidrug resistant). In the conventional view of drug resistance, one or several cells in the tumour population acquire genetic changes that confer drug resistance (FIG. 2a). These cells have a selective advantage that allows them to overtake the population of tumour cells following cancer chemotherapy. Based on the tumour-stem-cell concept, an alternative model posits that the cancer stem cells are naturally resistant to chemotherapy through their quiescence, their capacity for DNA repair, and ABC-transporter expression (FIG. 2b). As a result, at least some of the tumour stem cells can survive chemotherapy and support regrowth of the tumour. In a third model of acquired resistance, drug-resistant variants of the tumour stem cell or its close descendants arise, producing a population of multidrug-resistant tumour cells that can be found in many patients who have recurrence of their cancer following chemotherapy (FIG. 2c). The same mechanisms that allow stem cells to accumulate mutations over time, producing the long-term consequences of exposure to irradiation or carcinogens, would then allow cancer stem cells to accumulate mutations that confer drug resistance to their abnormally developing offspring[1]. As an example,

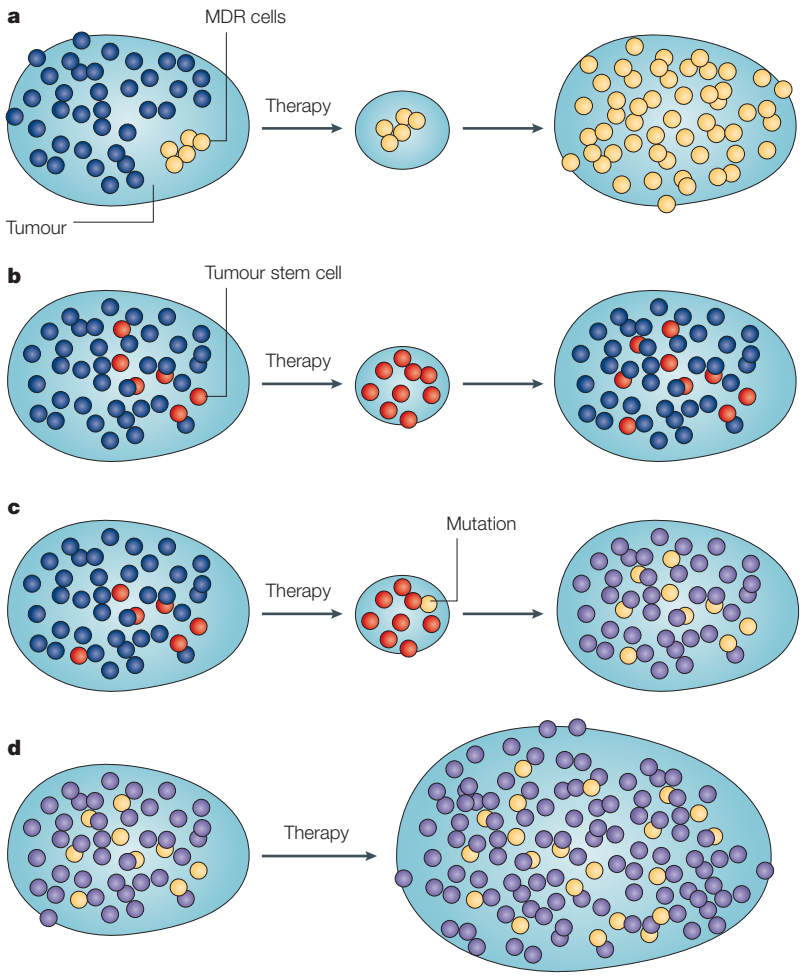


Figure 2 | **Models of tumour drug resistance. a** | In the conventional model of tumour-cell drug resistance, rare cells with genetic alterations that confer multidrug resistance (MDR) form a drug-resistant clone (yellow). Following chemotherapy, these cells survive and proliferate, forming a recurrent tumour that is composed of offspring of the drug-resistant clone. **b** | In the cancer-stem-cell model, drug resistance can be mediated by stem cells. In this model, tumours contain a small population of tumour stem cells (red) and their differentiated offspring, which are committed to a particular lineage (blue). Following chemotherapy, the committed cells are killed, but the stem cells, which express drug transporters, survive. These cells repopulate the tumour, resulting in a heterogeneous tumour composed of stem cells and committed but variably differentiated offspring. **c** | In the 'acquired resistance' stem-cell model, the tumour stem cells (red), which express drug transporters, survive the therapy, whereas the committed but variably differentiated cells are killed. Mutation(s) in the surviving tumour stem cells (yellow) and their descendants (purple) can arise (by mechanisms such as point mutations, gene activation or gene amplification), conferring a drug-resistant phenotype. As in model **a**, the stem cell with the aquired mutations could be present in the population before therapy. **d** | In the 'intrinsic resistance' model, both the stem cells (yellow) and the variably differentiated cells (purple) are inherently drug resistant, so therapies have little or no effect, resulting in tumour growth.

genetic alterations such as those that upregulate *ABCB1* expression in human leukaemia and lymphoma cells could have originated in the stem cell[55,56]. In a final 'intrinsic resistance' model, both the stem cells and the variably differentiated cells are inherently drug resistant, so therapies have little or no effect, resulting in tumour growth (FIG. 2d). An example of the latter is an intrinsically resistant cancer such as renal-cell cancer, in which *ABCB1* is expressed in all cells and contributes to chemotherapy tolerance. In this case, the resistance phenotype of the cancer stem cell persists in the committed, abnormally developing progenitors that comprise the proliferative pool of cancer cells.

So in the cancer-stem-cell model of drug resistance, tumours have a built-in population of drug-resistant pluripotent cells that can survive chemotherapy and regrow. Again, a parallel with normal stem cells can be found in stem-cell-driven recovery of normal tissues following chemotherapy. The rapid relapse observed with some tumours, at times within one cycle of chemotherapy, has a normal-tissue parallel in the repopulation of the bone marrow by normal haematopoietic stem cells and the recovery of the mucosa of the gastrointestinal tract, both of which usually occur within one 3-week cycle. Similarly, tumour recurrences that occur months to years after an original response to chemotherapy can be modelled on the slower recovery that is observed with hair follicles[57,58].

Although it is therapeutically attractive, the hypothesis that the intrinsic properties of stem cells alone provide the basis for drug resistance might be too simplistic. Recent studies of imatinib (Glivec) resistance in patients with leukaemia provide an example of how ABC-transporter-mediated efflux in stem cells could facilitate, but not be solely responsible for, the acquisition of acquired mechanisms of drug resistance. Imatinib has been recently shown to be both a substrate and inhibitor of ABCG2, making it susceptible to efflux by a stem cell that expresses this ABC transporter[59–61]. The initial studies that reported imatinib-resistant leukaemia cells described 'acquired' mutations in the kinase domain of ABL in patients with CML or with acute lymphoblastic leukaemia associated with t(9;22)(q34;q11). These findings indicate that although the expression of drug transporters by the cancer stem cell might provide some level of drug resistance, an acquired mutation in ABL could confer higher levels of drug resistance. Although these mutations might have arisen during therapy, their existence before the administration of imatinib has not been excluded. Indeed, pre-existing mutations that confer resistance to imatinib have also been described in a subset of patients[62,63]. These findings are reminiscent of the Goldie–Coldman hypothesis, proposed more than 20 years ago, that a small percentage of cells in a population harbouring intrinsic mutations confer drug resistance[64]. The Goldie–Coldman hypothesis would theorize that the cell acquiring the mutation is the stem cell.

Although the expression of ABC transporters could render stem cells resistant to drugs, it is not the sole determinant of resistance, as the DNA-repair capacity of the cell and the reluctance to enter apoptosis could be equally or more important. Generally regarded as quiescent and non-dividing, stem cells would be expected to be inherently refractory to drugs that target either the cell cycle or rapidly dividing cells. To the extent that quiescence is an important mechanism of drug resistance in stem cells, agents will have to be developed that are effective in non-dividing cells. For example, studies with imatinib have shown that blocking BCR–ABL-positive cells at the G1/S

Table 2 | **ABCB1 inhibitors**

Inhibitor	Limitations	Toxicity	Cancer tested	Clinical benefit in clinical trials?	References
Verapamil	Low potency	Hypotension	Multiple myeloma Breast cancer NSCLC SCLC	No Yes Yes No	88 89 90 91
Quinidine	Low potency	Gastrointestinal disturbance	Breast cancer	No	92
Cyclosporine A	Pharmacokinetic interaction	Nephrotoxicity	AML Multiple myeloma	Yes No	93 94
Valspodar (PSC833)	Pharmacokinetic interaction	Ataxia	AML	No*	95,96
Biricodar (VX710)	Pharmacokinetic interaction	Not known	None	Not known	97–100
Elacridar (GF120918)	Not known	Not known	None	Not known	101
Laniquidar (R101933)	Not known	Not known	None	Not known	102
Tariquidar (XR9576)	Not known	Not known	None	Not known	69
Zosuquidar (LY-335979)	Not known	Not known	None	Not known	103
ONT-093 (OC-144-093)	Not known	Not known	None	Not known	104
CBT-1	Not known	Not known	Not known	Not known	105

*Two randomized trials in acute mylogenous leukaemia (AML) demonstrated no overall benefit[95,96]; however, one trial indicated a benefit in the subset of patients with functional drug-transport activity[96]. NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.

boundary *in vitro* had no significant impact on the ability of imatinib to induce apoptosis, indicating that imatinib is effective in non-dividing cells[65–68].

Overcoming drug resistance

By inhibiting the main transporters of chemotherapy drugs, it was thought that drug resistance could be avoided and tumour cells eliminated. Therefore, much effort has been devoted to the development of inhibitors of ABC transporters. First-generation compounds included drugs identified as ABCB1 inhibitors, such as verapamil and cyclosporine, that were in clinical use to treat other diseases. These inhibitors were combined with a range of chemotherapy regimens for many cancers[30] As the results were not convincing, subsequent clinical trials were attempted with second-generation inhibitors such as PSC 833 and VX-710 (TABLE 2). The results of these trials were largely negative, failing in some cases because of pharmacokinetic interaction between the chemotherapeutic agent and the ABCB1 inhibitor. These studies might also have failed because of the presence of additional transporters, such as ABCC1 and ABCG2, that were not targeted by the inhibitor. Yet, although the results of these trials were negative, correlative studies did show that transport by ABCB1 could be inhibited. Efflux activity was assessed with a radionuclide-imaging agent (^{99m}Tc-Sestamibi), confirming that some human tumours have ABCB1 activity that can be suppressed with the ABCB1 inhibitors VX 710, PSC 833 and tariquidar (XR9576)[69–72]. The increased ^{99m}Tc-Sestamibi retention in the entire tumour following treatment with tariquidar indicates that the transporter-expressing phenotype of the cancer stem cell persists in the committed, abnormally developing progenitors that comprise the proliferative pool of cancer cells.

As cancer stem cells express drug transporters that make them resistant to many chemotherapy agents, anticancer strategies should include efforts to target these cells with their special properties. Clinical studies have attempted to overcome drug resistance through combination therapies in which a cytotoxic drug was given along with an ABC-transporter inhibitor. In a new paradigm, transport inhibitors might be thought of as 'tumour stem cell sensitizing agents' that allow the most crucial and most drug-resistant cells in a tumour to be destroyed. Skeptics could argue persuasively that ABCB1 inhibitors have shown very limited effectiveness in clinical trials. However, one could reply that clinical trials with these inhibitors have not focused on targeting cancer stem cells. Rather, they have determined response rates by measuring the reduction in size of tumours that express a particular drug transporter (usually ABCB1). If the stem cells are the main mediators of drug resistance, however, ABC inhibitors would not necessarily reduce tumour burden immediately, but efficacy could be observed using alternative end points, such as the frequency of relapse or the time to relapse. A skeptic would counter that these effects would surely have been reported in the trials conducted so far, if ABCB1 inhibitors did indeed destroy cancer stem cells. However, it is possible that the cytotoxic drugs or ABC inhibitors tested were inefficient in killing cancer stem cells. An inhibitor of drug transport might be most beneficial when combined with an anticancer agent that specifically targets the stem cells, such as imatinib, which targets the leukaemia stem cells that carry the BCR–ABL fusion protein. Another potential reason that clinical trials involving drug-transport inhibitors have not proven successful is that the wrong transporter was inhibited.

Box 1 | **New therapeutic opportunities**

ABCG2 inhibitors
Administration of ABCG2 inhibitors either before or during chemotherapy might help eliminate tumour stem cells. Two compounds (GF120918 and tariquidar) that inhibit both ABCG2 and ABCB1 are already approved for clinical studies. Additional ABCG2 inhibitors are in development.

ABCG2 antibodies
Antibodies against ABCG2 or other stem-cell markers might be useful in killing tumour stem cells. These antibodies could be used to deliver toxins or radioisotopes. The antibodies might also be used in diagnostics to detect tumours, visualize metastasis, or monitor therapy response or relapse.

Stem-cell inhibitors
Stem-cell self-renewal and survival requires signalling from a range of molecules through specific cell-surface receptors. A potential stem-cell inhibitor is cyclopamine, a compound that inhibits the Hedgehog–Patched receptor signalling protein Smoothened. Inhibiting such receptors and signalling molecules might preferentially inhibit tumour stem cells.

Immunotherapy
Several clinical protocols involve the activation of a patient's immune cells against his/her cancer cells, or the transplant of bone-marrow stem cells from a donor to kill the tumour cells in the recipient. Purified tumour stem cells from a patient could be lethally irradiated and used to 'immunize' the patient or to activate the donor's immune cells against the tumour stem cells.

Most of the studies evaluating cells with the SP phenotype have shown that stem cells overexpress ABCG2, rather than ABCB1, which has been the transporter targeted in most clinical studies[49]. To properly evaluate the latter possibility it will be important to develop an inhibitor for ABCG2. The compound fumitremorgin C (FTC) is a natural product that specifically inhibits ABCG2 (REF. 73). However this compound is toxic to cells, as well as to mice, and is not thought to be suitable for clinical studies. Chemically synthesized derivatives of FTC such as Ko143 have been developed, and several of these show high specificity and low toxicity[74]. In mice, these compounds have been shown to sensitize mouse tumour cells to drugs. Studies with Ko143 have also shown that inhibition of ABCG2 allows for a greater absorption of certain drugs across the intestine[74]. In addition, the compound GF120918 is an ABCB1 inhibitor that has been shown to inhibit ABCG2 *in vitro* and apparently also *in vivo*[75].

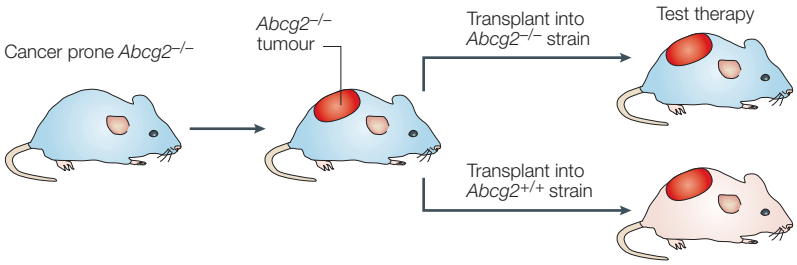


Figure 3 | **Mouse models for testing tumour-stem-cell therapies.** Numerous cancer-prone mouse strains exist and they can be crossed with mouse strains with disruptions in the *Abcg2* gene. Tumours and tumour stem cells (red) that arise in these mice would not express ABCG2, so therapies could be tested either in the *Abcg2*$^{-/-}$ (blue) or a wild-type strain (pink). This would allow for the true impact of complete ABCG2 suppression to be assessed, as the tumour cells would lack the gene encoding this protein. If the absence of ABCG2 improves the chemotherapy, this would give justification for further experiments with ABCG2 inhibitors. Experiments with *Abcg2*$^{+/+}$ tumours are more complicated, as the inhibition of ABCG2 could lead to severe toxicity of the normal stem cells of the mouse.

The identification of potent, specific and non-toxic inhibitors of ABCB1, ABCG2 and ABCC1 is required before the full effects of blocking these transporters can be determined. However, this might be difficult to accomplish *in vivo* without the destruction of normal stem cells — especially of haematopoietic stem cells — that depend on the expression of drug transporters to survive drug therapy. Stem-cell-driven tissue repopulation not only mediates regrowth of tumours, but also mediates regrowth of normal tissues in the adult, including the bone marrow, gastrointestinal tract and hair follicles. Whether a 'therapeutic window' exists that would allow the destruction of cancer stem cells but not normal stem cells remains to be determined.

Future directions

One cannot deny the appeal of explaining the pool of drug-resistant cells and the problem of chemotherapy resistance in terms of the existance of a relatively quiescent stem-cell population armed with multiple drug transporters. But, how does this model fit in the context of the clinical problem of drug resistance? Unfortunately, for most drug-resistant cancers, including kidney, pancreatic and colon cancers, the problem is not that a few cells survive but, rather, that only a few cells die in response to chemotherapy. For this vexing problem, the stem-cell model of drug resistance at present has little applicability. But for cancers that respond to chemotherapy with an apparent clinical complete response, only to relapse months or years later, this stem-cell model of drug resistance has more appeal. Admittedly, students of this problem will quickly point out that this hypothesis is not new; that the only thing new is that the term stem cell is used to describe those cells that were previously refered to as resting in G0 (REFS 76,77).

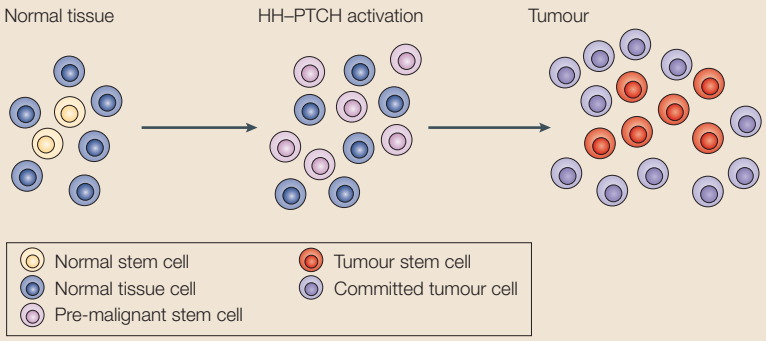
To get the best results, future stem-cell models will need to have various qualities. First, they will need to define stem cells by tumorigenicity or clonogenicity (that is, their capacity for long-term self-renewal), and

Box 2 | **Hedgehog signalling and cancer**

The Hedgehog molecules (SHH, IHH and DHH) are important signalling proteins in the development of embryonic stem cells and in the differentiation of many tissues[79]. Hedgehog (HH) binds to the cell-surface receptor Patched (PTCH) and signals through the Smoothened (SMO) and GLI proteins. This pathway has a clear role in tumour formation in patients with nevoid basal-cell carcinoma syndrome, in which *PTCH* mutations have been described[80–82]. Additional members of the HH pathway have also been found to be tumour suppressors or oncogenes[78]. Recently, components of the HH–PTCH pathway have been shown to be disrupted or overexpressed in a large number of tumours, including sporadic medulloblastomas, breast, prostate, stomach, colon and pancreatic cancers[83–87].

Most sporadic medulloblastomas have either germline *PTCH* mutations or *PTCH* silencing through methylation. Treatment of medulloblastomas with the SMO-inhibitory compound cyclopamine resulted in reduced proliferation and changes in gene expression consistent with differentiation[77]. Small-cell lung tumour cell lines show high expression of *SHH*, and their growth can be inhibited by SHH antibodies or cyclopamine[85]. Similarly high levels of HH expression and HH–PTCH pathway activation have been found in oesophageal, stomach, pancreatic, prostate and biliary tumours and in cell lines. Treatment with cyclopamine led to regression of pancreatic and prostatic tumours in mice, providing a model system for therapeutic development[84,87].

HH overexpression could lead to the unregulated growth of tissue stem cells (see figure). This would result in a pre-malignant lesion in which abnormal stem-cell growth drives hyperproliferation. These unregulated stem cells would be the target for genetic events that drive the stem cells into the formation of tumour stem cells. Continued evolution of the tumour stem cells could occur to give rise to metastatic cells or further drug resistance.



In the above figure, normal stem cells (blue) undergo transformation to a stem cell with abnormal HH–PTCH signalling. This cell (orange) proliferates abnormally, but is pre-malignant. Subsequent genetic events give rise to a tumour stem cell (red) that can generate additional stem cells with abnormal signalling and tumour cells that are committed but incompletely differentiated (purple)[68].

not merely by presence of the SP phenotype. Second, they will need to reconcile the observation that many normal tissues and well-differentiated tumours have high levels of the same ABC transporters found in stem cells. Third, they will have to explain how over time, a repopulated tumour acquires increasing drug resistance. Fourth, they will need to delineate the 'plasticity' of cells in a tumour (that is, the extent to which cells downstream of stem cells can acquire the capacity to self renew). Last, they will have to address the problem of intrinsic resistance, where all cells are refractory to drug therapy, not just the small population of stem cells. Theoretical considerations aside, an exciting aspect of these new lines of inquiry into cancer is that there are many tools at hand to quickly allow crucial questions to be answered (BOX 1). Excellent approaches have been developed to isolate and grow stem cells. Initial analysis of gene expression in these cells reveals many genes that are either over- or underexpressed in tumour stem cells. Isolation of stem cells from different types of tumour will allow determination of the similarity or differences of these molecular profiles among different stem cells. This could lead to improved diagnostic tools to detect pre-malignant lesions and tumours, as well as targeted therapies, such as antibodies, directed against tumour stem cells. Agents that suppress stem-cell growth might be useful chemopreventive agents in individuals with a high cancer risk, much as we inhibit oestrogen production or action in those at risk of breast cancer.

Abcb1-, *Abcg2*- and *Abcc1*-null mice have been generated, and all are viable. If the transporters encoded by these genes are required for the protection of stem cells, then mice that lack these genes might have a higher susceptibility to tumorigenesis from certain mutagenic chemicals. Such studies could lead to the development of interesting new cancer models. To better assess the role of the ABCG2 and ABCB1 transporters in chemotherapy, it is possible to study tumours that develop in mice that lack these genes. This will allow the isolation of cancer stem cells that lack these transporters and the direct testing of the ability of current and future drugs to kill cancer stem cells (FIG. 3). Mice with conditional knockouts of these genes can be developed to allow the tumour stem cells to form in the presence of the transporters, and the genes can then be deleted before the start of the chemotherapy.

One additional pathway important for the growth and differentiation of stem cells is the Hedgehog–Patched (HH–PTCH) pathway. Studies of the HH–PTCH pathway in tumours provide support for the importance of tumour stem cells in cancer[78] (BOX 2), indicating that proliferation of normal stem cells is regulated by signals from surrounding normal cells. Transformation of these stem cells can lead to a pre-malignant stem cell with abnormal HH expression or deficient PTCH activity. Such cells can grow in an unrestrained manner, leading to local proliferation. Additional genetic events give rise to a tumour stem cell that can generate more tumour stem cells as well as mature tumour cells. This model leads to specific hypotheses that can be tested as well as new avenues for therapeutics. For example, is the HH overexpression that is seen in many tumour cell lines a property of the stem cells or of all the cells in the population? Do drugs like cyclopamine, which targets the HH–PTCH pathway, slow tumour growth by inhibiting stem cells? Can ABCG2 inhibitors be combined with these drugs to provide higher levels of chemotherapy drugs to tumour stem cells, without toxicity to normal stem cells?

The existence of tumour stem cells might underlie the intractable nature of many human cancers, explaining why conventional cancer therapy fails in many patients. Normal stem cells evolved in the presence of radiation and multiple environmental toxins, and their ability to expel these toxins is essential for survival in adverse conditions. Although the existence of tumour

stem cells helps explain some previously recognized phenomena, they are far from being fully understood. The models advanced must be considered as 'works in progress' that will ultimately require adjustments and refinements. However, the fact that we can now identify, purify and propagate cancer stem cells allows the development of new strategies to improve targeted therapies in cancer.

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Competing interests statement
The authors declare no competing financial interests.

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