TIMELINE

Leukaemia stem cells and the evolution of cancer-stem-cell research

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Abstract | Many cancers seem to depend on a small population of 'cancer stem cells' for their continued growth and propagation. The leukaemia stem cell (LSC) was the first such cell to be described. The origins of these cells are controversial, and their biology — like that of their normal-tissue counterpart, the haematopoietic stem cell (HSC) — is still not fully elucidated. However, the LSC is likely to be the most crucial target in the treatment of leukaemias, and a thorough understanding of its biology — particularly of how the LSC differs from the HSC — might allow it to be selectively targeted, improving therapeutic outcome.

It is now half a century since bone-marrow reconstitution experiments, following lethal irradiation in mice, first indicated the existence of the haematopoietic stem cell (HSC)[1,2] — a cell first postulated to exist by Artur Pappenheim as early as 1917 (REF. 3). Although this cell population is still not fully characterized, its discovery awakened the field of stem-cell biology. The recent discovery of many more tissue-specific stem cells[4–7] has kept this field of study at the forefront of biological research.

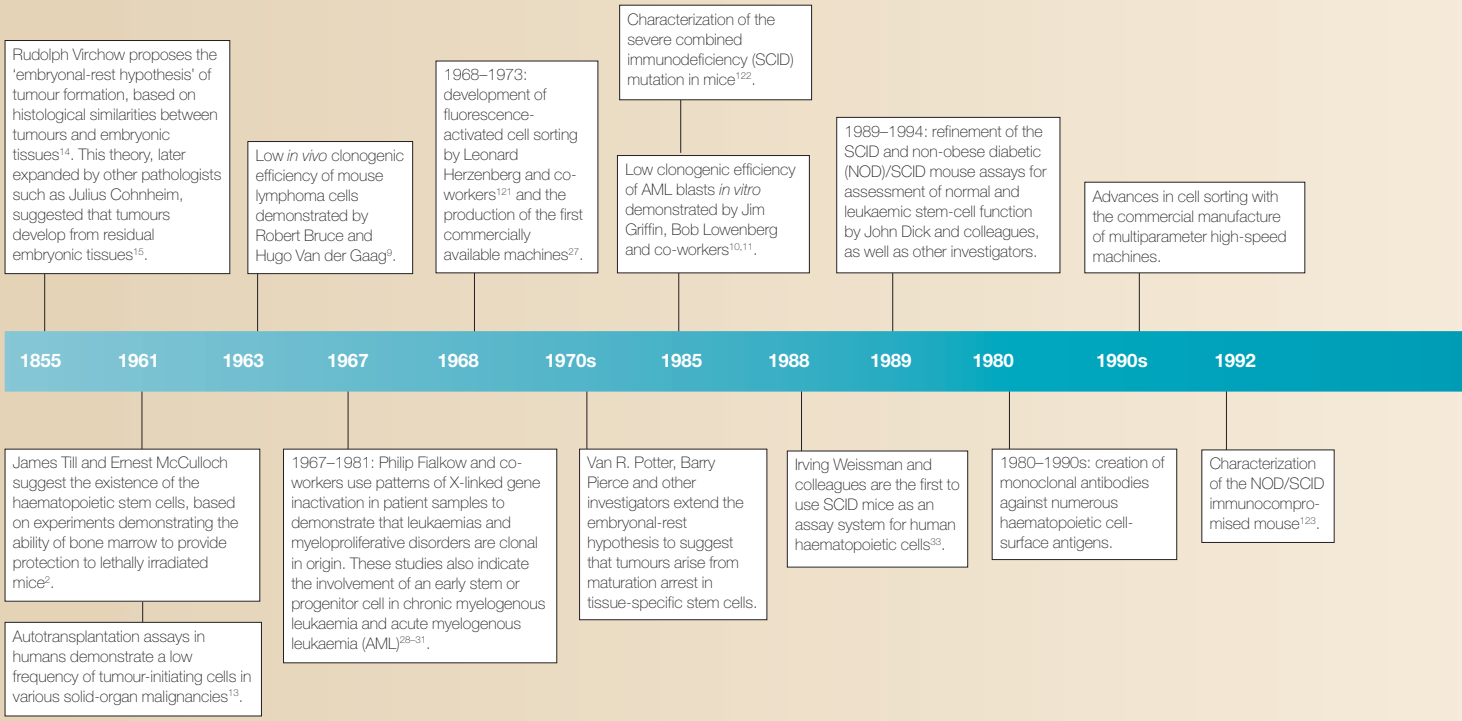
At around the same time that the existence of the HSC was postulated, observations were reported of the heterogeneous potential of tumour cells to self-renew both *in vitro* and *in vivo*. For example, in 1973, Ernest McCulloch and colleagues observed that only 1 in 100 to 1 in 10,000 murine myeloma cells had the ability to form colonies *in vitro*. In 1963, Robert Bruce and colleagues showed that only 1–4% of transplanted murine lymphoma cells formed colonies in spleens of recipient animals[8,9]. This low clonogenic potential was also observed in human leukaemia cells by Jim Griffin and colleagues in 1985, who reported that acute myelogenous leukaemia (AML) blasts (a blast is a haematopoietic tumour cell with very primitive morphology, indicative of an acute leukaemia or lymphoma) formed colonies at low frequency in methylcellulose[10,11].

Also at this time, it was found that solid-organ cancer cells vary in their ability to proliferate in similar assays. In standard soft-agar assays, where colony formation provides a surrogate for transformation, Anne Hamburger and Sydney Salmon found that only 1 in 1,000 to 1 in 5,000 cells isolated from solid tumours (such as lung, ovarian and brain tumours) were capable of forming colonies[12]. In addition, in 1961, experiments (which now could not be justified ethically) carried out by Chester Southam and Alexander Brunschwig showed that tumour cells that had been harvested from patients with disseminated malignancy and then injected subcutaneously into the same patients led to a low frequency of tumour formation, and that tumours were only initiated when over 1,000,000 cells were injected[13]. These observations led the investigators to speculate that the entire population of a tumour's cells might arise from a few so-called 'cancer stem cells'[12].

A normal stem cell is defined by its dual properties of self-renewal and multilineage differentiation potential, and continuously repopulates the mature cells of the organ system that it serves. Although homeostatic pressures can dictate that a stem cell undergoes symmetric division to produce two daughter cells that are either both stem cells or both progenitor cells (this outcome depends on need) stem cells are defined by their ability to divide asymmetrically. Through this process, the division of a stem cell results in the formation of two daughter cells — one of which is another stem cell, and the other of which is a committed progenitor that is capable of further differentiation and proliferation but lacks the ability to self-renew. A cancer stem cell would function in a similar way to sustain the growth and spread of tumours while repopulating the distinct cell types represented within the tumour. However, a cancer stem cell would not be subject to the same intrinsic and extrinsic controls as normal stem cells.

This 'cancer-stem-cell hypothesis' represents a modern-day interpretation of the proposal made by pathologists such as Rudolph Virchow and Julius Cohnheim ~150 years ago (TIMELINE) that cancer results from the activation of dormant embryonic-tissue remnants[14,15]. This 'embryonal-rest hypothesis' of cancer was based on the histological similarities between the developing fetus and certain types of cancer, such as teratocarcinomas[14], and the observation that both tissues have an enormous capacity for both proliferation and differentiation, albeit aberrant differentiation in the case of tumours. There is substantial evidence in the literature to support this mechanism in paediatric cancers; nephrogenic rests (abnormally retained embryonic kidney precursor cells arranged in clusters) that antedate the development of Wilm's tumour can be detected by ultrasound during gestation[16], and certain mutations can be detected at birth that are associated with a predisposition to paediatric leukaemias. These mutations include the somatic *TEL–AML1* (REF. 17), *MLL–AF4* (REF. 18), *AML–ETO* (REF. 19) and *OTT–MAL* translocations, as well as constitutional trisomy 21 (REF. 20). Van R. Potter and Barry Pierce

Timeline | **The evolution of cancer-stem-cell research**



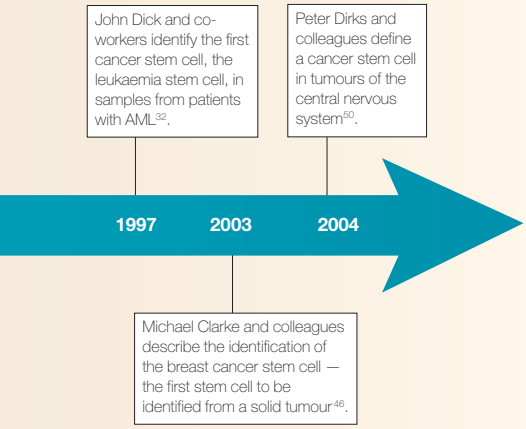
updated these ideas in the 1970s and 1980s, describing cancer as the maturation arrest of tissue-determined stem cells[21–23]. However, formal proof of these hypotheses would have to await further technological advances.

So, it seems that tumours are composed of a heterogeneous population of cells, within which resides a small population of cancer stem cells that are exclusively responsible for the growth and propagation potential of the whole tumour. However, an alternative hypothesis, the stochastic model, could also explain the heterogeneous potential of tumour cells to self-renew. This model predicts that all tumour cells have the potential to self-renew and recapitulate the tumour, but that the probability that any particular tumour cell enters the cell cycle and finds an environment permissive for growth in an assay of tumorigenesis is low[24]. To differentiate between these two models it is necessary to define distinct populations of cells within tumours, based on surface immunophenotypic or functional characteristics, to purify these populations to homogeneity and to develop long-term assays of their functional ability. The functional assessment of a cancer stem cell requires not only the ability to form a new tumour, but to recapitulate precisely the phenotype of the initial disease (BOX 1).

The haematopoietic system is amenable to *ex vivo* analysis, and, in the late 1980s and 1990s, developments in antibody technology allowed investigators — such as Irving Weissman and colleagues — to define the ontogeny of this system, based on surface immunophenotype in humans and mice (for a comprehensive review see REF. 25). These studies identified a large number of phenotypic markers that are associated with defined lineages and developmental stages of haematopoietic cells, and concomitantly allowed the annotation of malignant haematopoietic ontogeny. Another technical advance that greatly facilitated the characterization of leukaemia and other cancer stem cells was the development of high-speed multiparameter flow cytometry. This is an automated technique that is used to identify and/or purify distinct cell populations, based on the ability of fluorescently labelled antibodies to bind cell-surface antigens. Following excitation with a laser, the fluorochrome-bound antibody emits light of a specific wavelength, allowing the detection and enumeration of cell populations that express the antigen. Furthermore, by applying an electric charge to deflect populations that are positive for a predefined fluorochrome, and therefore antigen, these populations can be collected for functional analysis[26,27].

Based on functional and immunophenotypic analysis of subpopulations of cells with modern technologies, cancer has become viewed increasingly as a stem-cell disorder, in which the continued growth and propagation of the whole tumour depends on a small subpopulation of self-renewing cancer stem cells. The HSC was the first adult somatic stem cell to be described. The existence of cancer stem cells was also first described in the haematopoietic system. The demonstration of a leukaemia-initiating cell, now commonly referred to as the leukaemia stem cell (LSC), in AML and other leukaemias, along with the subsequent identification of cancer stem cells in breast and central-nervous-system (CNS) tumours, provides evidence for the broad applicability of the cancer-stem-cell model.

It is plausible that malignant stem cells share a number of biological features that are different from their normal-tissue counterparts and that these might be exploited for therapeutic benefits. As malignant haematopoieisis is well characterized and amenable to laboratory and clinical investigation, further investigation of the biology of the LSC could provide a paradigm for all cancer stem cells and could improve the therapy of both leukaemias and other solid-organ cancers. In addition,



a better understanding of the relationship between normal and cancer stem cells might provide insights that allow us to harness self-renewal programmes for regeneration of selected tissues without the need for embryonic tissue.

Demonstration of the LSC

Elegant experiments by Philip Fialkow and colleagues, in which they used patterns of inactivation in X-linked genes, had previously shown that leukaemias such as chronic myelogenous leukaemia (CML)[28] and AML[29], together with preleukaemic diseases such as the myeloproliferative disorders[30], are clonal in origin. Analyses of X-inactivation patterns in various haematopoietic lineages also indicated that early stem and progenitor cells are involved in the development of CML and AML[31]. However, it was not until advances in the identification and separation of discrete tumour-cell populations, and the availability of appropriate assays (BOX 1), that the existence of an LSC was first demonstrated. In a series of seminal experiments in 1997 by investigators based at the University of Toronto — where James Till and Ernest McCulloch had first shown the radioprotective effects of mouse bone marrow[2] — the LSC for AML[32] was first identified.

Box 1 | **Functional assays for normal and malignant stem cells**

For a malignant or normal cell to be considered a stem cell, it must be able to self-renew and entirely reconstitute its particular organ system over an extended period of time. The stem cell achieves this through a process of asymmetric division to generate one new stem cell and one daughter cell, which undergoes differentiation. Stem cells are also capable of symmetrical division to form two daughter cells or two stem cells — the outcome depends on extracellular factors. To test the self-renewal potential of a cell population, it is necessary to purify this cell population to functional homogeneity. For example, for a normal haematopoietic stem cell, this would involve reconstitution of all hematopoietic lineages, whereas a leukaemic stem cell must accurately recapitulate the leukaemia phenotype. Although the differentiation potential of a cell can often be assayed *in vitro*, self-renewal can only be measured *in vivo* (shown in diagram). This requires the transfer of the potential stem-cell population to an immunologically permissive recipient, where the cells could undergo self-renewal and differentiation. *In vivo* assessment of the self-renewal potential of human stem cells has relied on xenotransplantation models, in which human normal or malignant stem cells are grafted into immunocompromised mice[124]. Severe combined immunodeficient (SCID) mice, which lack both B and T cells, engraft both normal haematopoietic stem cells (HSCs; left side of diagram), as well as leukaemia stem cells (LSCs; right side of diagram) isolated from patients with acute myelogenous leukaemia (AML) and chronic myelogenous leukaemia (CML)[35,36]. SCID mice can also be crossed with non-obese diabetic (NOD) mice, which lack natural-killer cell activity and have other immune deficiencies, such as defects in macrophage activity and complement activation. These NOD/SCID progeny can engraft both normal and malignant human haematopoietic cell types and sustain serial transplantation[125,126] — a necessary requirement for the assessment of long-term self-renewal potential. Transplantation experiments in NOD/SCID mice have therefore become standard assays for the assessment of the self-renewal and reconstitution potential of normal and malignant HSCs.

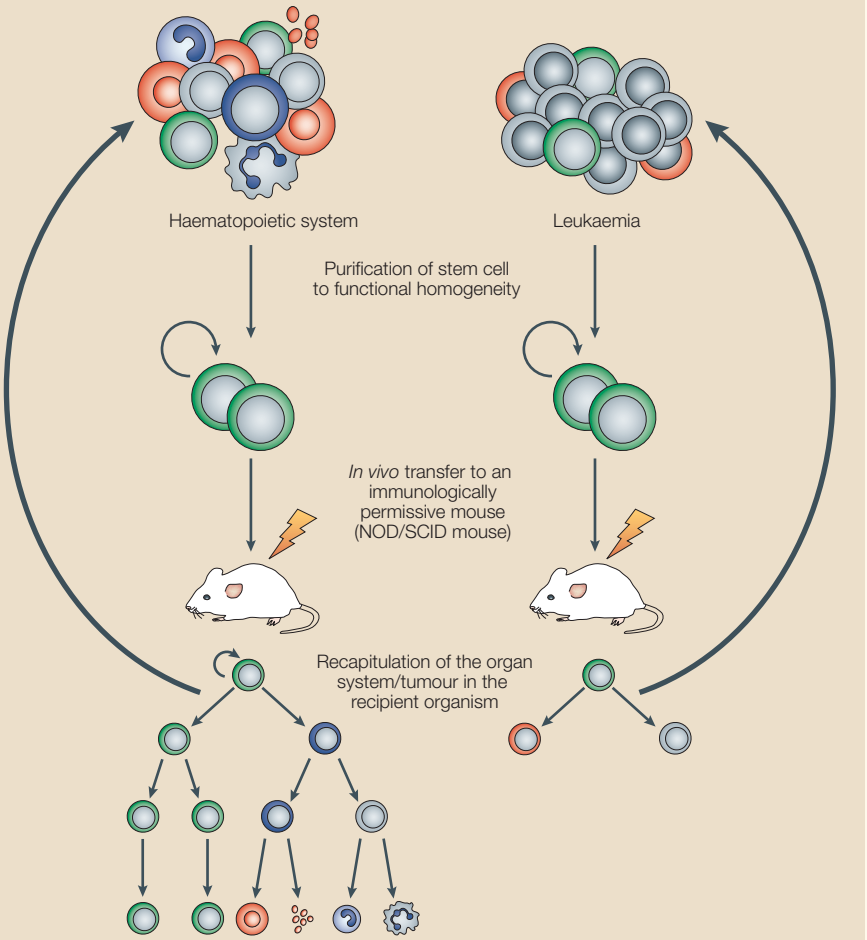


Table 1 | **Markers of malignant and normal haematopoietic ontogeny**

Marker	Normal cell expression pattern	Malignant cell expression pattern	Description/function
CD10	Precursor B cells; some mature B-cell subsets; neutrophils; some non-haematopoietic stromal cells	Common ALL cells; follicular lymphoma cells; Burkitt's lymphoma cells	Neutral endopeptidase; metalloendopeptidase
CD19	Precursor and mature B cells	B-cell leukaemia and lymphoma cells	Signal-transduction molecule that regulates B-lymphocyte development activation and differentiation
CD20	B cells; dendritic cells	B-cell lymphoma cells	Regulator of cell-cycle progression
CD34	Haematopoietic stem/progenitor cells; endothelial cells	AML and ALL cells; bulk and leukaemia stem cells	Sialomucin-like adhesion molecule; involved in cell adhesion
CD38	Myeloid, B- and T-cell progenitors; monocytes; activated B and T cells; plasma cells	Non-clonogenic AML cells; multiple myeloma cells	ADP-ribosyl cyclase; regulator of cell activation and proliferation
CD45RA	B cells; monocytes; macrophages; activated T cells	B-cell lymphoma cells	Restricted leukocyte common antigen; regulator of leukocyte activation
CD71	All proliferating cells — particularly erythroid progenitors	All proliferating cells	Transferrin receptor — mediates iron entry into the cell
CD90 (THY1)	Haematopoietic stem cells; T cells; fibroblasts; stromal cells	Some AML cells and occasional cells from patients with ALL; fibroblast tumours	Potentially contributes to haematopoietic and neural stem-cell differentiation
CD123 (IL-3Rα)	Myeloid progenitors	AML cells, both bulk and leukaemia stem cells	IL-3Rα — the low affinity binding subunit of the IL-3 receptor, which binds to IL-3 in association with α-subunit
CD138	B cells; plasma cells	Multiple myeloma cells	Syndecan-1 — an extracellular matrix receptor
HLA-DR	B cells; antigen-presenting cells; myeloid progenitor cells	Expressed on most cells from patients with AML (except patients with acute promyelocytic leukaemia); most B-cell neoplasms; occasionally by T-ALL cells and melanoma cells	Human leukocyte antigen class II gene cluster — processes and presents antigen, and also interacts with $CD4^+$ T cells

ALL, acute lymphoblastic leukaemia; AML, acute myelogenous leukaemia.

John Dick and colleagues were pioneers in developing and refining the severe combined immunodeficient (SCID) mouse and the non-obese diabetic (NOD)/SCID mouse xenotransplantation stem-cell assay systems, which were first described by Weissman and colleagues[33] (BOX 1). Dick showed that normal human stem cells, as well as unfractionated AML, acute lymphoblastic leukaemia (ALL) and CML cells isolated from patients, could engraft and proliferate in SCID mice, and that the progeny of these cells could be detected by flow cytometry[34–36]. Moreover, all mature lineages, with the exception of T cells, were detected in mice that were recipients of normal stem cells, whereas leukaemic cells recapitulated human leukaemias in recipient mice. These findings indicated that tumour cells exist that fulfilled the criteria to be called 'stem cells'.

These investigators named the primitive cell responsible for the induction of normal haematopoiesis in the recipient mice the 'SCID repopulating cell' (SRC), and the cell responsible for induction of leukaemia in mice the 'SCID leukaemia-initiating cell' (SL-IC)[36]. Furthermore, a surface phenotype similar to the $SRC^-CD34^+CD38^-$ (TABLE 1) was demonstrated for the SL-IC in one patient with the M1 subtype of AML. However, limitations of the SCID mouse assay system did not allow confirmation of the SL-IC phenotype from other AML subtypes or allow the authors to test long-term self-renewal of this population. In the SCID system, large cell inoculums are required to ensure engraftment, and serial transplantation to subsequent generations of recipient mice to test for long-term self-renewal is not possible.

The development of the NOD/SCID-leukaemia model obviated these drawbacks, and allowed the separation of leukaemia cells into subpopulations that could be evaluated for engraftment and serial-transplantation potential. Cells derived from seven patients representing each subtype of AML (according to French-American-British classification standards), were separated into $CD34^+CD38^+$ and $CD34^+CD38^-$ fractions. These subpopulations were injected intravenously into sublethally irradiated NOD/SCID mice that received regular injections of human cytokines. The mice were assessed at 4–8 weeks for engraftment, based on human-specific DNA sequences. Human cells from the bone marrow of transplant recipients were then isolated, based on the expression of human form of CD45 (TABLE 1), and transplanted into secondary recipients. These experiments showed that the capacity to transfer human AML to recipient mice resided exclusively within the $CD34^+CD38^-$ fraction. Furthermore, these cells had the same capacity to induce all subtypes of AML, except for M3 (the most differentiated subtype of AML, acute promyelocytic leukaemia). The leukaemias that developed in the secondary recipients closely resembled the human cancer, demonstrating that LSCs have long-term self-renewal capabilities and also determine the stage of the differentiation block during leukaemogenesis.

Based on these findings, the authors proposed a hierarchical organization for AML that is similar to normal haematopoiesis (FIG. 1). In this model, the LSC (or SL-IC, as the authors named it) is responsible for both self-renewal and the production of clonogenic leukaemic progenitors that have proliferative capacity, but not the capacity of self-renewal. Based on similarities in the organization of the respective systems and phenotypic similarities, they also proposed that the HSC was the most likely target for transformation into an LSC. Further experiments from the laboratories of Craig Jordan and Donna Hogge have refined the immunophenotype of the LSC in AML to be $CD34^+CD38^-CD90^-$ interleukin 3 receptor $(IL\text{-}3R)^+CD71^-$ human leukocyte antigen $(HLA)\text{-}DR^-CD117^-$ (REFS 37–40) (TABLE 1).

Similar functional studies have been performed using primary ALL cells. In ALL cells that carry the *BCR–ABL* fusion gene, the SL-IC is also defined by the $CD34^+CD38^-$ immunophenotype[41]. In B-cell precursor ALL, the SL-IC is $CD34^+$ but lacks expression of the more mature B-lymphoid markers CD10 and CD19 (REF. 42).

Another cancer type that is associated with the *BCR–ABL* fusion is CML, which is also considered to be a stem-cell disorder. Expression of the BCR–ABL fusion transcript has been observed in many mature haematopoietic lineages as well as the $CD34^+$ stem-cell compartment[43,44]. In addition, the observation that CML cells have the potential to generate acute leukaemias of various lineages during disease progression to blast crisis (the last of the three phases of CML)[43] also indicates the transformation of a pluripotent cell, that is, an HSC. However, recent evidence from Irving Weissman's laboratory indicates that the CML stem-cell compartment might be dynamic when it progresses to blast crisis[45]. In patients with myeloid CML blast crisis, self-renewal properties were observed *in vitro* within the granulocyte monocyte progenitor (GMP) population, a compartment that normally lacks the potential for self-renewal, indicating that this progenitor compartment might contain the stem cells responsible for the expansion of acute leukaemic blasts on transformation. These data demonstrate that the LSC hierarchy might change or operate on different levels during the progression of leukaemia, with the accompanying acquisition of further mutations.

Application of the principles of stem-cell biology by John Dick and others recently led investigators such as Michael Clarke and Peter Dirks to propose a similar paradigm for solid-tumour development. However, stem cells have been much more difficult to identify in non-haematopoietic cancers, because fewer well-developed phenotypic markers and definitive assay systems are available. Nonetheless, significant progress has been made in the identification of cancer stem cells in both breast and CNS tumours[46–50]. Breast cancer stem cells were first identified by Clarke and colleagues in 2003, based on the expression of surface markers and their potential to form tumours after injection into the mammary fat pad of NOD/SCID mice. Cells with the phenotype of epithelial-specific antigen $(ESA)^+$ Lineage marker $(Lin)^-CD24^{-/low}CD44^+$ (TABLE 1) were found to generate tumours that were histologically similar to those of primary breast tumours in mice when as few as 100 cells were transplanted[46]. Cells from these NOD/SCID recipients were re-purified, and cells with the same phenotype were found to reform tumours when transplanted into secondary and tertiary recipients. These experiments demonstrated the long-term self-renewal capacity of these cells, thereby fulfilling the definition of a cancer stem cell from breast tumours.

In 2003, there were three separate reports of the characterization of CNS tumour stem cells, from the laboratories of Peter Dirks, Harley Kornblum and Dennis Steindler. Previous studies had shown that neural stem cells (NSCs) form neurospheres in culture[4], and that this function makes it possible to isolate these cell populations from brain tumours for further analysis. The tumour NSCs can differentiate into cells that have characteristics of both neurons and glial cells, self-renew *in vitro* at higher levels than normal NSCs, and grow and differentiate in neonatal rat brains[47–49]. Cells with these properties all express $CD133^+$ (REF. 47), a marker of NSCs, and a recent report from Peter Dirks' laboratory in Toronto demonstrated that only this $CD133^+$ subpopulation has the ability to regenerate identical brain tumours in NOD/SCID mice. Furthermore, these tumours could be serially transplanted[50]. It is likely that, as suitable markers and assay systems become available, more solid-tumour cancer stem cells will be described.

Characterization of the LSC

Although the existence of a malignant stem-cell population has been demonstrated for a number of cancer types, the nature and origins of this cell remain to be fully elucidated. It has been proposed that the target cells for malignant transformation are adult somatic stem cells[24], and that oncogenic mutations appropriate the capacity for self-renewal inherent in these cells. Stem cells persist throughout adult life and undergo an increased number of cell divisions, and therefore have a greater opportunity than short-lived cells to acquire the minimum number of mutations required for malignant transformation[51–53].

Phenotypic evidence indicates that somatic stem cells are target cells for malignant transformation. Stem cells and tumour cells express similar levels of telomerase[54], which is required to maintain telomere length and prevent replicative senescence. The human LSC population that generates AML

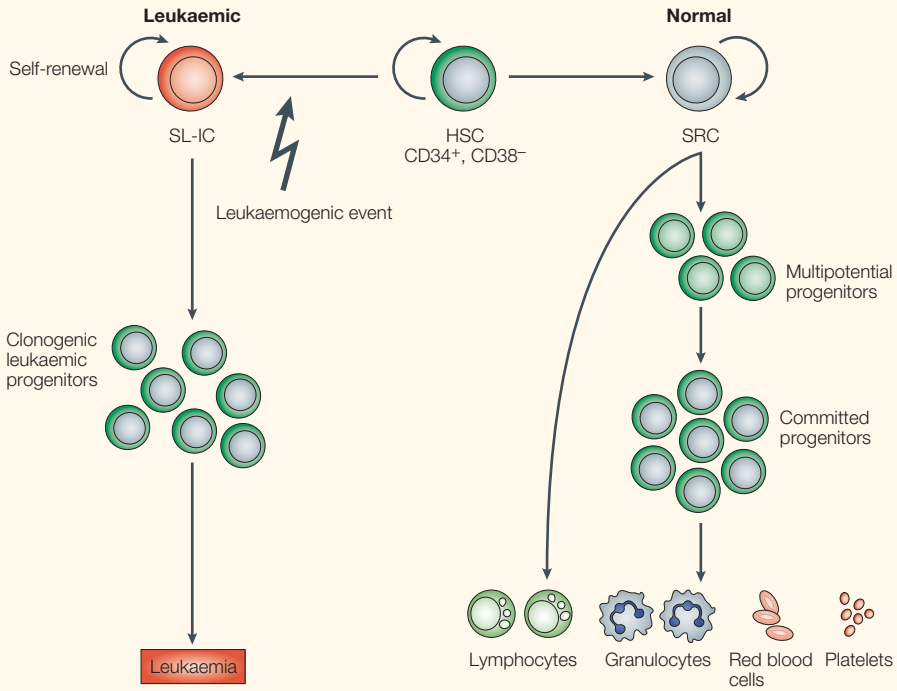


Figure 1 | **Acute myelogenous leukaemia forms a stem-cell hierarchy.** There are functional and phenotypic similarities between the normal haematopoietic stem cell (HSC) and the leukaemia stem cell (LSC) that initiates AML. Like the normal haematopoietic system, leukaemia cells are believed to be derived from HSCs, which are $CD34^+CD38^-$. During normal cell haematopoiesis (right), the HSC gives rise to the severe combined immunodeficiency (SCID) repopulating cell (SRC), which is capable of self-renewal and production of all mature lineages through a succession of multipotent and committed progenitors. During leukaemogenesis (left), the LSC (or SCID leukaemia-initiating cell, SL-IC) expresses some shared surface phenotypic markers with the HSC. It also has the capacity for producing both the clonogenic leukaemic progenitors and the non-clonogenic blast cells, which make up the bulk of the leukaemia. Figure adapted from REF. 32 © (1997) Macmillan Magazines Ltd.

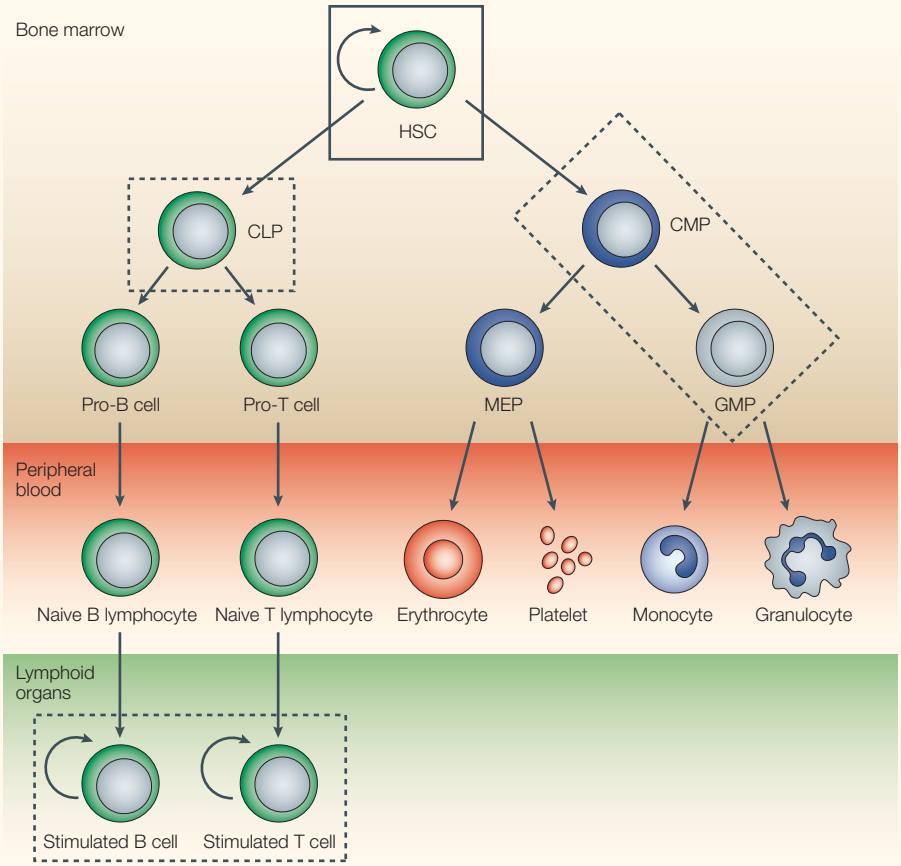


Figure 2 | **Ontogeny of haematopoietic cells and candidate leukaemia stem cells.** Within the haematopoietic system, the haematopoietic stem cell (HSC) has self-renewal potential and multipotent differentiation potential. Its progeny include multipotent common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). In turn, these progenitors give rise to progenitors that have more limited differentiation potential, Pro-B and Pro-T cells, megakaryocyte erythroid progenitors (MEPs) and granulocyte monocyte progenitors (GMPs). In the myeloid lineage, MEPs give rise to mature erythrocytes and platelets in the peripheral blood. GMPs give rise to monocytes and the various granulocyte lineages. In the lymphoid lineages Pro-B and Pro-T cells give rise to mature (naive) B and T lymphocytes and then stimulated B and T cells, respectively, following exposure to antigen. Cellular compartments that are known to be, or might be, potential targets for the generation of leukaemia stem cells (LSCs) are indicated by solid or dashed boxes, respectively. Not all of these cell types have self-renewal properties (which is indicated with a curved arrow). Mutations are therefore required to confer properties of self-renewal to these cell types, and to lead to LSC formation and leukaemogenesis.

in the NOD/SCID mouse model has a similar phenotype ($CD34^+CD38^-$) to that of normal human HSCs, which induce multilineage human haematopoiesis when engrafted into NOD/SCID mice[36], and also to the cell compartment that is enriched for human HSC[55]. Furthermore, cytogenetic abnormalities that are consistently associated with certain leukaemias have been detected in this cell compartment in patients with AML, CML and ALL[43,56–58]. In addition, the t(9;22) (Philadelphia chromosome), which results in the *BCR–ABL* gene rearrangement associated with CML, can be detected in cells of the myeloid, erythroid, megakaryocytic and B-lymphoid lineages. This indicates that the initial transformation occurred in a cell with multi-lineage differentiation potential, such as an HSC[43,44]. Moreover, a recent mouse model has shown that genetic inactivation of the AP-1 transcription factor JUNB can cause a myeloproliferative, CML-like disease in mice only when JUNB is inactivated in the HSC compartment[59]. Another recent study demonstrated that LSCs that have different capacities for long-term self-renewal[60] could be identified following serial transplantation of lentivirally labelled human AML cells into NOD/SCID mice. These data are consistent with the maintenance of a normal HSC hierarchy in the LSC compartment, and further implicate the HSC as a candidate cell for transformation by leukaemia-inducing oncogenes.

However, committed progenitor cells that do not have the capacity to self-renew might also be transformed through the activation of oncogenic pathways that re-establish properties of self-renewal (FIG. 2). Proof of principle for this concept has recently been established in mouse models of leukaemia[61,62]. In these experiments, populations of progenitor cells that lack self-renewal properties were purified by flow cytometry and transduced with either the *MLL–ENL* or *MOZ–TIF2* oncogenes, both of which were originally cloned from human leukaemias and generate leukaemia on retroviral expression in mouse models. Both of these fusion genes also involve transcriptional regulators — MLL is a histone methyltransferase involved in chromatin remodelling[63], and both MOZ and TIF2 are transcriptional co-activators[64]. Transduction with either oncogene leads to the restoration of self-renewal properties in these progenitors in *in vitro* assays — such as the ability to form colonies in serial methylcellulose plates in the absence of stromal support, and to grow in liquid culture — properties that are not even demonstrated by normal HSCs. These transduced progenitors were also able to generate AML when transplanted into mice, and the resulting leukaemias could be transplanted to secondary recipients. These properties could not be attributed solely to insertional mutagenesis by the retroviral vector, because transduction with transformation-defective mutants of *MOZ–TIF2* and *MLL–ENL* did not confer this phenotype[61,62]. Furthermore, self-renewal is not conferred by all leukaemia-associated oncogenes — transduction with *BCR–ABL* does not change the self-renewal properties of committed progenitor populations[62].

Corroborating evidence for progenitor cells as targets for transformation has also been observed in human leukaemia cells. A recent report of the organization of the LSC compartment following the progression of CML to its acute, blast-crisis phase indicates that a committed progenitor population might be the target for further oncogenic mutations that re-confer properties of self-renewal. For example, GMPs purified from patients with blast crisis CML have self-renewal properties *in vitro*[45]. These data also raise the possibility that ongoing acquisition of second mutations that confer properties of self-renewal to progenitor cells could also contribute to a hierarchy of LSCs that have different long-term self-renewal properties[65]. This has been shown for AML cells[60]. Although *in vivo* confirmation of self-renewal in the GMP population of patients in the

blast-crisis stage of CML is required, this report further implicates transcriptional targets of β-catenin as the mediators of self-renewal in this system. Transduction of normal GMPs with β-catenin restores *in vitro* self-renewal to this population, and the inhibition of nuclear β-catenin localization in blast-crisis GMPs, through lentiviral expression of the specific inhibitor axin, inhibits self-renewal.

Therefore, considerable evidence exists that committed progenitors might be transformed to generate LSCs. Although the HSC is an attractive candidate for the cell of origin of the LSC, it is also the most dangerous cell type for transformation. In metazoan biology, stem cells are the sole guardians of self-renewal in adult tissues, and would therefore be expected to have evolved sophisticated inhibitory machinery to prevent uncontrolled self-renewal. However, these mechanisms would not be expected to function in daughter cells, which lack self-renewal properties. It seems that there are oncogenic mutations that are tolerated by the HSC, such as *BCR–ABL*, and that are passed on to the progeny of the mutated stem cell. These mutations might be tolerated because they have little or no effect on the stem cell itself, and confer their main biological impact on downstream progeny. However, mutations that increase self-renewal and or impair differentiation (alone or in cooperation with other mutations) might be toxic or not tolerated by the HSC. Alternatively these mutations could simply be constrained, such that the aberrant self-renewal that they confer can only alter the capabilities of downstream progeny. Mutations that affect self-renewal and proliferation appear to be associated primarily with acute haematological malignancies. So, it might be (for the above reasons) that the stem cell is actually less likely to acquire these mutations, or to manifest biological alterations that are secondary to these mutations. In either situation, the net result will be the same, in that the LSC in acute disorders frequently lies downstream of the stem-cell compartment.

Regulating stem-cell self-renewal

Relatively little is known about the molecular mechanisms of self-renewal in normal or malignant stem-cell populations[24]. However, it seems likely that there will be at least some degree of overlap in the regulation of self-renewal in normal and malignant stem cells. Genes and pathways implicated in the molecular control of self-renewal include products of the *Bmi1* (also known as *PCGF4*) and other polycomb genes[66–68], the WNT–β-catenin pathway[45,69–71], the Notch[72,73] and SHH[74,75] signalling pathways, as well as the HOX family of transcription factors[76–78]. The WNT–β-catenin, BMI1/polycomb and SHH signalling pathways have been expertly reviewed elsewhere[79–81].

Notch family. Notch family members are developmental morphogens[82]. In the year 2000, the Notch family members were shown to be expressed in self-renewing tissue, and activation of Notch signalling was subsequently shown to potentiate self-renewal of both HSCs[72,73] and NSCs[83,84]. Notch signalling is also required for T-cell commitment[85]. There are four mammalian Notch genes, each of which encodes a single-pass transmembrane receptor. Notch signalling is initiated by the binding of the cognate Jagged or Delta-like ligands, resulting in metalloproteinase (γ-secretase) cleavage in the extracellular portion of the receptor with release of intracellular Notch (for an extensive review of Notch function see REF. 86) (FIG. 3). Intracellular Notch translocates to the nucleus, forms a multimeric transcriptional complex with the transcription factor CSL and co-activators of the mastermind-like (MAML) family, and activates the transcription of Notch target genes involved in context-dependent processes such as T-cell differentiation and self-renewal.

Notch family members were then shown to have activity as oncogenes or tumour suppressors, depending on the cellular context[86,87]. The role of Notch as an oncogene was best characterized in T-cell ALL (T-ALL). In 1991, a small fraction of patients with T-ALL were found to express a constitutively activated form of NOTCH1, as a consequence of chromosomal translocation involving the T-cell receptor β-subunit[88]. Subsequent studies showed that NOTCH1 signalling caused T-ALL in mouse models[89,90].

A recent study from the laboratories of Jon Aster and Tom Look reported that more than 50% of patients with T-ALL carried somatic activating point mutations of NOTCH1 (REF. 91). These mutations affected either the heterodimerization domain, leading to cleavage and liberation of intracellular NOTCH1 (ICN), or resulted in loss of function of the PEST domain, which targets ICN for degradation by the proteasome (FIG. 3). Both mutations, which often occurred on the same allele, independently led to the increased activation of the Notch-signalling pathway, supporting a cooperative effect between the mutations in NOTCH1 activation.

The increase in Notch-pathway signalling activity appears to both induce T-cell differentiation and increase self-renewal properties in primitive haematopoietic progenitors, resulting in T-ALL. A similar heterodimerization mutation in the *Caenorhabditis elegans* Notch homologue *glp-1* has been described and leads to a massive proliferation of germ cells[92]. Interestingly, inhibitors of the proteolytic enzyme γ-secretase have already been developed, because of the involvement of this enzyme in the production of amyloidogenic peptides in patients with Alzheimer's disease. These γ-secretase inhibitors might therefore be of therapeutic value in patients that have T-ALL associated with NOTCH1-activating mutations, and also as probes for self-renewal potential conferred by NOTCH1. Therapeutic trials of γ-secretase inhibitors in patients with T-ALL are now under development, and represent an initial step in molecularly targeting the self-renewal potential of leukaemia stem cells.

HOX family. The homeodomain-containing transcription factors of the HOX family have also been found to be important in haematopoietic development, leukaemogenesis and self-renewal. They were initially widely studied as regulators of pattern formation and tissue identity during embryogenesis in both *Drosophila melanogaster* and mammalian systems by investigators such as Robert Krumlauf [93]. In 2002, HOX genes were shown to be expressed in haematopoietic precursors, with preferential expression in self-renewing HSCs, and are downregulated following differentiation[94]. Their importance in normal blood formation and maintenance has been demonstrated in gene-targeting studies — abnormalities in multiple haematopoietic lineages were observed in mice with disruptions in individual HOX genes[95,96]. Moreover, the overexpression of specific HOX genes, such as *HOXB4*, is associated with the expansion of the HSC compartment *ex vivo*[78] and with an increase in competitive self-renewal following transplantation experiments in mice. Similar findings were reported for both *HOXB4* and *HOXA9* (REFS 77, 97, 98).

The relationship between only certain HOX genes and leukaemogenesis is also a mystery — transplantation of mice with bone marrow that overexpresses *Hoxa9* will eventually lead to leukaemia, whereas recipients of bone-marrow cells that overexpress *Hoxb4* do not develop leukaemia. However, in the 1990s, many HOX genes were directly linked to the pathogenesis of acute leukaemia[99]. Chromosome translocations that fuse *HOXA9* or *HOXD13* to *NUP98*, which encodes a component of the nuclear pore, were observed in AML cells[100,101]. *HOX11* overexpression, resulting from its

translocation to promoter regions of the T-cell receptor locus, was also described in T-ALL cells[102]. Moreover, *HOX11* and *HOX11L2* were found to be frequently upregulated in T-ALL cells and to confer favourable or poor prognosis, respectively[103].

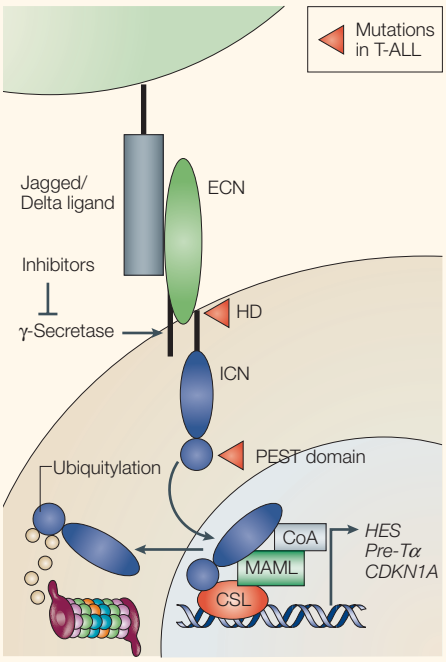


Figure 3 | **The Notch signalling pathway.** Notch family members are transmembrane receptors and developmental morphogens. In adult tissue, activation of the NOTCH1 pathway mediates context-specific functions, such as self-renewal and T-cell differentiation. NOTCH1 signalling is initiated by the engagement of extracellular portions (ECN) of NOTCH1 with its ligands, which are members of the Jagged/Delta family. This binding induces metalloprotease-dependent cleavage of the NOTCH1 heterodimerization domain (HD) with terminal cleavage that is dependent on γ-secretase activity. This process releases intracellular NOTCH1 (ICN), which translocates to the nucleus to form a multimeric transcriptional factor complex with the transcription factor CSL and co-activators of the mastermind-like (MAML) family. These recruit additional co-activators, such as p300 and PCAF (CoA), to activate the transcription of target genes such as *HES*, *pre-Tα* and *CDKN1A*. The transcriptional programmes that are initiated are thought to mediate T-cell differentiation and increase the self-renewal that is associated with NOTCH1 signalling activity. ICN is targeted for degradation by the polyubiquitylation of sequences in its PEST domain. Mutations (red arrows) in the HD and PEST domain of NOTCH1 have recently been associated with T-cell acute lymphoblastic leukaemia (T-ALL). These mutations are believed to activate NOTCH1 signalling by increasing NOTCH1 cleavage and liberation of ICN, as well as by increasing NOTCH1 protein stability by decreasing proteosomal degradation.

HOXA9 overexpression is now the single most highly correlated factor with poor prognosis in patients with AML[104]. Several other members of the HOX gene family, such as *Hoxb3* (REF. 105), *Hoxb8* (REF. 106) and *Hoxa10* (REF. 107), have also been associated with leukaemogenesis in mouse models. These findings indicate a role for HOX genes in leukaemia induction, and their role in HSC self-renewal indicate that they might also mediate LSC self-renewal.

The central role of HOX deregulation in leukaemia is further emphasized by the role of upstream regulators such as MLL and the CDX proteins in leukaemia. Targeted-disruption experiments have demonstrated that the *Mll* gene in mice is required for the maintenance of *HOX* gene-expression patterns during development[108]. Rearrangement of MLL with more than 40 partner genes has been reported in acute leukaemia (reviewed in REFS 109,110) and transformation by MLL fusion proteins is thought to be mediated by their altered transcriptional properties[63], with dysregulation of HOX gene expression as a crucial event[111]. Another recently described gene family, CDX, also regulates expression of HOX genes[112]. The CDX genes are mammalian homologues of the *Drosophila melanogaster caudal* gene, a master regulator of embryonic body segmentation[113]. CDX genes are also implicated in human leukaemogenesis, with the description of a t(12;13) in human AML[114]. This chromosomal rearrangement juxtaposes the *TEL* and *CDX2* loci and is thought to result in the overexpression of *CDX2* from the *TEL* locus, and expression of CDX2 induces leukaemia in murine retroviral transduction models[115].

Future directions

Decades of research into the pathways that control cellular self-renewal indicate alterations in gene transcription as a common end point. However, the specific transcriptional programmes that are activated by these pathways are not well understood. The leukaemia-associated fusion genes *MOZ–TIF2* and *MLL–ENL* confer properties of self-renewal to committed myeloid progenitors[61,62]. They are able to transform cells by altering their transcriptional programme[64,116]; and presumably restore self-renewal properties to progenitor cells through similar mechanisms. Moreover, the restoration of *in vitro* self-renewal properties in GMPs isolated from patients with blast-crisis CML appears to be associated with increased transcriptional activity of β-catenin[45], albeit with unknown gene targets.

Taken together, these reports indicate that the self-renewal properties that are required for leukaemia induction and maintenance are restored (or augmented) by transcriptional programmes induced by certain oncogenes. However, as leukaemogenesis is a multistep process [117], the transcriptome of leukaemia stem cells will demonstrate changes that reflect a number of oncogenic mutations. In future experiments, mouse models may simplify matters and reveal many of the changes that result from the overexpression of a single oncogene in a homogeneous cellular context, and might provide a powerful prospective platform to elucidate the transcriptional programmes involved in self-renewal. Candidate self-renewal genes could be identified through microarray analysis, and could be validated using functional assays of mouse and human LSCs and HSCs. Comparisons of LSC and HSC gene-expression patterns might identify similarities and differences in molecular self-renewal mechanisms used by normal and malignant stem cells. It is also likely that the WNT, polycomb, HOX, SHH and Notch pathways form important hubs in the network of self-renewal transcriptional programmes. Further study of the downstream targets of these self-renewal pathways, and of any potential interactions between these pathways[80] might be used to identify crucial effectors of self-renewal.

The identification of additional genes that regulate self-renewal might also provide rational targets for therapeutic intervention — particularly if their requirement is more critical for self-renewal in LSCs than HSCs. Understanding the pathogenic mechanisms of leukaemia stem cells might also provide insight into the reasons that chemotherapeutic regimens can markedly reduce tumour burden, but result in relapse at a later stage. It is likely that chemotherapeutics only target the bulk, non-clonogenic tumour cells and spare the leukaemia stem cells, allowing for recrudescence of tumour following cessation of therapy[79]. Because unlimited self-renewal is an absolute prerequisite for tumour growth[118], inhibition of this property should have a marked effect on cancer progression and recurrence following, or in conjunction with, standard therapies. This would be a paradigm shift in the treatment of leukaemia, away from targeting the blast cells and towards the targeting of the LSC. This approach could increase the incidence of remission and decrease the incidence of relapse. A challenge to this approach is to find a way to specifically target cancer-cell self-renewal

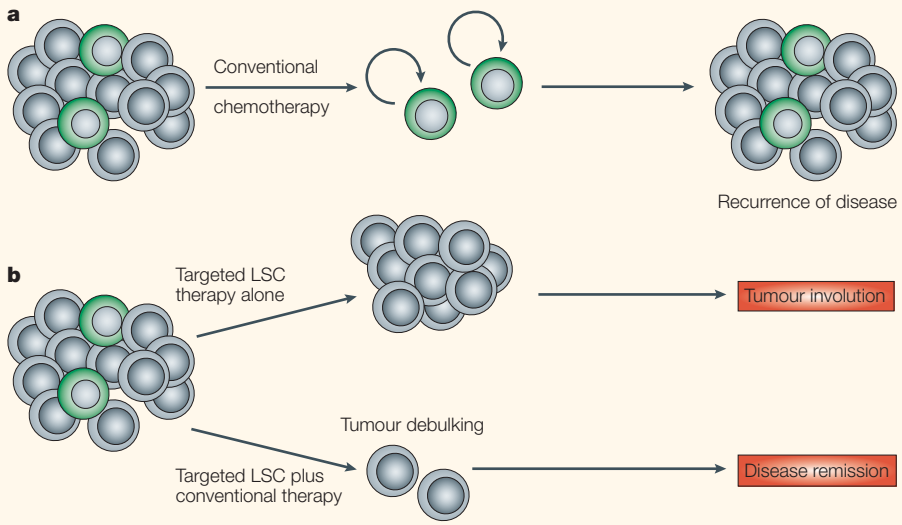


Figure 4 | **Targeting leukaemia stem cells. a** | At present, treatment for leukaemia uses chemotherapeutic agents that target all leukaemia cells (grey), based on properties such as their increased proliferation and entry into the cell cycle. However, it is likely that this approach spares the population of leukaemia stem cell (LSCs; green), which are responsible for the continued growth and propagation of the tumour. In many instances, this leads to recurrence of the disease. **b** | A greater understanding of LSC biology will allow us to design therapeutic agents that specifically target the LSC populations. Such therapies used alone, or in combination with conventional chemotherapeutic agents that reduce tumour burden, should lead to tumour involution or disease remission, respectively. Both of these approaches could improve both initial response rates and overall survival, through a decrease in the relapse of disease.

pathways without toxicity to normal cells[119,120] (FIG. 4). Trials with γ-secretase inhibitors, which target the Notch signalling pathway in T-ALL cells, might provide some insight into the therapeutic window in targeting self-renewal.

As there are many functional similarities between the LSC and solid tumour stem cells, it is likely that malignant stem cells will share a number of molecular mechanisms by which they control fundamental cellular processes such as self-renewal, cell-fate determination and apoptosis. The further characterization of the LSC will therefore provide paradigms of the biology of malignant stem cells that can be applied to solid-organ tumours and might also lead to improved therapeutics for these malignancies.

Building on the seminal observations of John Dick and others, a picture is emerging in which tumours have a similar hierarchical development and architecture as the normal-tissue counterpart, including the existence of a multipotent stem cell and an aberrant differentiation programme. Not only is this relevant in the haematopoietic system in which it was first defined, but also in several solid tumours. Indeed, it is plausible that such a hierarchy is present in all tumours. Because there is an enticing congruence between normal and malignant stem-cell function, it is probable that if we understand one, we will have major insights into the other.

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Competing interests statement
The authors declare no competing financial interests.

Online links

DATABASES
The following terms in this article are linked online to:
Cancer.gov: http://cancer.gov/
acute lymphoblastic leukaemia | acute myelogenous leukaemia | chronic myelogenous leukaemia
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ABL | *BCR* | *HOX11* | *HOX11L2* | *HOXA9* | *HOXB4* | *HOXD13* | *NOTCH1* | *NUP98*

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INNOVATION

High-intensity focused ultrasound in the treatment of solid tumours

James E. Kennedy

Abstract | Traditionally, surgery has been the only cure for many solid tumours. Technological advances have catalysed a shift from open surgery towards less invasive techniques. Laparoscopic surgery and minimally invasive techniques continue to evolve, but for decades high-intensity focused ultrasound has promised to deliver the ultimate objective — truly non-invasive tumour ablation. Only now, however, with recent improvements in imaging, has this objective finally emerged as a real clinical possibility.

The 1990s witnessed an explosion in minimally invasive alternatives to open surgery for localized malignancy. Quite apart from the inherent attractions of new technology, the incentives behind this movement are plain. Open surgery is associated with significant morbidity and with mortality, and causes suppression of a patient's immune system, which in turn can lead to the risk of perioperative metastatic tumour dissemination. Patients themselves usually complain of postoperative pain and recovery can be lengthy. Laparoscopic surgery might be more acceptable to patients, and leads to a quicker return to work, but usually takes longer, and both operative morbidity and mortality are broadly comparable with open surgery.

Other minimally invasive techniques use a range of energy-based methods for *in situ* tumour destruction. Apart from radiotherapy, these include radiofrequency ablation, laser ablation, cryoablation (BOX 1) and high-intensity focused ultrasound (HIFU). In principle, where surgery usually aims to remove a tumour with an adequate normal-tissue margin, if a minimally invasive technique can destroy the equivalent tissue volume, then outcome in terms of disease-free survival should be at least equal. If operative mortality is avoided, then outcome could even be better. In fact, taking the example of interstitial laser ablation for isolated colorectal liver metastases, data are now emerging to support this assertion[1].

Treatment with HIFU is the only one of these alternatives to surgery that is truly non-invasive. Theoretical advantages of this lack of invasiveness are that there is no risk of tumour seeding along a needle track, which has been reported after procedures such as percutaneous ethanol injection[2] and radiofrequency ablation[3], and there is no risk of haemorrhage from visceral or vascular puncture, which can occur during any of the minimally invasive procedures described in BOX 1. A high-energy focused ultrasound beam is directed harmlessly across the skin and intervening tissues towards the target tumour. Only at the focus of the beam is the energy level great enough to cause a temperature rise sufficient for instantaneous cell death. The mechanism of action of HIFU is not tumour-specific and so a wide range of tumour types can be targeted. In addition, in contrast to ionizing radiation, treatment can be given more than once as there is no upper limit of tissue tolerance to repeated ultrasound exposure. There are very few side effects of treatment, and serious adverse events are rare. As a result, HIFU treatment with palliative intent, aimed either towards symptom or local tumour control can also be seriously contemplated for patients with poor prognoses.

In several centres worldwide, HIFU is now being used clinically to treat solid tumours (both malignant and benign), including those of the prostate[4], liver[5,6], breast[7], kidney[8], bone and pancreas, and soft-tissue sarcoma[6]. This has only been the case for the past 5 years, so, perhaps with the exception of prostate cancer, the evidence base for long-term efficacy is far from mature. However current data are very encouraging and the role of HIFU in oncology is likely to expand as devices become more widely available.

How does HIFU work?

The term 'ultrasound' refers to mechanical vibrations above the threshold of human hearing (16 kHz). Medical ultrasound is generated by applying an alternating voltage across a piezoelectric material such as lead zirconate titanate. Such materials oscillate in thickness at the same frequency as the driving current. The resulting ultrasound wave propagates through tissues, causing alternating cycles of increased and reduced pressure (compression and rarefaction, respectively). Most of us are familiar with diagnostic ultrasound, which usually uses frequencies in the range of 1–20 MHz. By contrast, frequencies of 0.8–3.5 MHz are generally used during the clinical applications of HIFU, and the energy levels carried in the HIFU beam are several orders of magnitude greater than those of a standard diagnostic ultrasound beam. In a way analogous to the focusing of light, ultrasound waves can be focused at a given point. The high energy levels carried in a HIFU beam can therefore be magnified further and delivered with precision to a small volume, while sparing surrounding tissues[9].