Stem Cells in Acute Lymphoblastic Leukaemia

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Abstract

There has been significant debate over the identity of cancer stem cell populations in acute lymphoblastic leukaemia (ALL), with different groups reporting seemingly contradictory results. The latest findings suggest that tumour-propagating capacity is found within a high percentage of ALL blasts and that these cells have diverse immunophenotypes, which suggests that ALL follows a stochastic cancer stem cell model – as opposed to a hierarchical model. Recent data add a layer of complexity to the tumour evolution process by showing that the leukaemia-propagating compartment consists of multiple genetically diverse subclones related by Darwinian-style evolutionary trees. Differences in the cell of origin may also affect tumour development. In this article, we discuss the applicability of cancer stem cell models to ALL in the context of these recent findings.

Keywords

Cancer stem cells, acute lymphoblastic leukaemia, clonal evolution, cell of origin

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In the early 1990s, pioneering work from John Dick's laboratory demonstrated that only a rare subset of malignant acute myeloid leukaemia (AML) cells could reconstitute the disease following successive xenotransplantations in mice.[1] This work was possible due to critical advances in haematopoietic stem cell (HSC) isolation based on surface marker expression profiles,[2] and provided the first experimental evidence for a concept that had been previously discussed for decades: the cancer stem cell (CSC). The CSC model contends that long-term tumour growth is driven by a population of cells with stem cell-like properties, resulting in the formation of tumours that are functionally and morphologically heterogeneous.

This heterogeneity can be explained by two CSC models. In the hierarchical model, CSCs are a biologically distinct subset of cells that both sustain the stem cell pool through self-renewal and give rise to progeny lacking extensive proliferative capacity. This model is analogous to the function of stem cells in normal tissue development. It follows from this that elimination of the CSC compartment will result in cessation of tumour growth. In contrast, the stochastic model contends that all cells within a tumour have the potential to act as CSCs and that functional heterogeneity is influenced by other factors. These could be either intrinsic (e.g., varying levels of particular transcription factors) or extrinsic (e.g., the tumour niche).

There is a substantial amount of evidence that the hierarchical model applies in some tumours. The initial findings of John Dick's laboratory, which have since been expanded upon,[3] showed that the long-term tumour-maintaining capacity in AML lies only within a rare subpopulation. Although other studies suggest the phenotype of these cells may be more diverse than initially thought,[4,5] the bulk of the evidence indicates that AML follows a hierarchical stem cell model. Xenotransplantation studies have also demonstrated that other solid tumours follow the hierarchical model, including breast,[6] colon[7,8] and brain[9] cancers. However, it appears that the model may not apply to all tumours, as the frequency of propagating cells in melanoma is very high (one in four)[10] and stem cell activity is associated with all cellular phenotypes[11] – although contradictory findings have been reported.[12]

Cancer Stem Cells in Acute Lymphoblastic Leukaemia

Acute lymphoblastic leukaemia (ALL) is a malignant disorder of lymphoid progenitor cells and the most common paediatric cancer. It is usually characterised by chromosomal translocations resulting in the formation of oncogenic fusion genes, which cooperate with other oncogenic mutations to induce leukaemia. Common examples include *TEL/AML1* (also known as *ETV6/RUNX1*), *BCR/ABL1* (Philadelphia chromosome [Ph+]) and *MLL/AF4*.[13]

Unlike in AML, where a hierarchical CSC model seems to apply, the question of which CSC model describes ALL has remained controversial. Attempts to investigate the issue have used transplantation of ALL cells into immunodeficient mice, a model pioneered by Kamel-Reid et al. in mice with severe combined immunodeficiency (SCID).[14] Initial findings appeared to show that the hierarchical model described the biology of childhood ALL, with only

sorted blasts displaying primitive immunophenotypes able to transfer precursor B cell ALL (B-ALL) to recipient animals.[15–17] More recently, Cox et al. reported that only immature CD133+CD19- and CD133+CD38- childhood B-ALL blasts can serially engraft non-obese diabetic (NOD)/SCID recipient mice and recapitulate the diagnostic immunophenotype.[18] There have also been studies showing leukaemia-propagating ability only within restricted populations in precursor T cell ALL (T-ALL).[19,20] Together, these data were suggestive of a rare, immature leukaemic stem cell (LSC) population in ALL.

However, further analysis has cast doubt on these findings. Work from our laboratory has involved intrafemoral injections of sorted B-cell precursor childhood ALL populations into immunodeficient mice, providing a more sensitive assay for human LSCs due to enhanced engraftment.[21,22] In contrast to previous results, we showed that CD34+CD19+, CD34-CD19+ and CD34+CD19- populations from high-risk ALL patients were able to engraft and reconstitute NOD/SCID mice over a series of sequential transplantations, suggesting that all these populations contained cells with stem cell-like activity. We expanded upon this using mice lacking natural killer (NK) cell activity (NOD/SCID IL2Rγ^{null}), which have increased immunodeficiency and thus improved engraftment of human cells. Use of this assay showed that CD19+CD20-/^{low} and CD19+CD20^{high} populations had leukaemia-propagating capabilities and could reconstitute the original patient immunophenotype. These results were observed with primary sorted cells as well as cells recovered from engrafted mice, showing that the results were not an artefact due to passaging human cells through mice. The identity of tumour-propagating populations is also likely to be dependent on the types of ALL studied, as previous work showed they are restricted to CD19+ fractions in standard-risk ALL.[23]

Our results are supported by data from Kong et al., who demonstrated that both CD34+CD19+CD38- and CD34+CD19+CD38+ cells from *MLL/AF4*-positive B-cell precursor ALL can propagate the leukaemia in NOD/SCID IL2Rγ^{null} mice.[24] Moreover, other studies have shown that fewer than 100 unsorted cells from poor outcome B-ALL patients are required to generate leukaemias in NOD/scid/IL2Rγ^{null} or NOD/SCID gamma (NSG) mice, which have further reductions in NK cell activity, thus demonstrating a high frequency of tumour-propagating cells in ALL.[25,26] One open question that needs to be addressed is whether stem cell frequency in ALL is solely model-dependent (as suggested by these results) and in principle every ALL blast can propagate the leukaemia, or whether stem cell frequencies vary between different ALL subtypes and may correlate with clinical outcome.

More recently, we continued using highly immunodeficient NSG mice to conclusively define the leukaemia-propagating populations.[27] Using samples from a range of different ALL subtypes, we have demonstrated engraftment potential in populations both positive and negative for CD34, CD19 and CD10, which define different stages of B cell development. In nearly all cases, limiting dilution assays showed high stem cell frequencies (1:40 to 1:2,900 cells) in both immature (CD34^{high} or CD20^{low}) and mature (CD34^{low} or CD20^{high}) blast populations. These populations could also transfer the leukaemia to secondary recipients and reconstituted the original immunophenotype, demonstrating a high degree of malleability in different blast populations. This finding argues against a hierarchical stem cell model in ALL and instead supports the stochastic model. We also showed that CD34^{high} and CD34^{low} blasts have similar expression of a candidate self-renewal gene signature, but differential expression of a B cell differentiation gene set, suggesting that the expression of candidate leukaemia stem cell genes is independent of the degree of blast maturation.

In summary, the results described above clearly demonstrate that there is no stem cell hierarchy in B-ALL. Instead, leukaemia-initiating potential lies within a wide array of blasts with diverse immunophenotypes, which is consistent with the stochastic model.

Effects of Experimental Models on Leukaemic Stem Cell Identification

The xenotransplantation of cell populations into immunodeficient mice has long been considered the 'gold standard' assay for identification of cells with tumour-propagating activity. Serial transplantation of these cells into secondary and tertiary recipients is able to demonstrate self-renewal, thus identifying the cells as CSCs. However, differences in xenograft models may explain some of the controversies over the involvement of LSCs in ALL. The studies that have only identified leukaemia-initiating activity in immature ALL populations have mainly used standard NOD/SCID mice as xenograft recipients, while those demonstrating tumourigenicity in a wider range of cell types have used more highly immunodeficient mice, such as NSG mice. This suggests that the different levels of immunosuppression in these mice may affect the identification of leukaemia-propagating cells, as conventional NOD/SCID mice have greater innate immunity than NSG mice (although the use of different mouse strains does not affect CSC detection in some tumours, such as pancreatic adenocarcinoma[28]).

Work by Taussig et al. has demonstrated that treating cells with anti-CD38 antibodies has an inhibitory effect on engraftment in NOD/SCID mice.[29] Furthermore, they showed that this effect is overcome by treating the mice with immunosuppressive antibodies and that, using this optimised model, CD38+ AML blasts do have stem cell activity. This casts doubt on previous studies that have used cell-sorted populations positive for CD38. Anti-CD33 antibodies have also been shown to inhibit leukaemic cell engraftment in NOD/SCID mice.[30] Another study has shown that only 15 % of primary leukaemic samples from B-ALL patients can successfully engraft SCID mice.[31] These factors raise the possibility that many assays using NOD/SCID mice underestimate the frequency of LSCs due to poor engraftment, potentially due to maintained innate immunity. Differences in the microenvironment of mice compared with humans, such as a lack of niche signals and the contribution of the stroma, may also both positively and negatively affect engraftment.

To summarise this section, it is likely that the differing conclusions on the role of stem cells in ALL reached by different groups result from limitations of the mouse models used. Both the degree of immunodeficiency and other uncharacterised factors within the bone marrow environment mean that some mouse strains are more suitable hosts for human cells than others. The standardisation of these models and the continued development of models with increased ability to engraft human cells, such as NSG mice, which also express human cytokines,[32] are likely to result in more of a consensus over the involvement of CSCs in ALL. The studies which have used the best available mouse models of human cell engraftment suggest that there is a high frequency of initiating cells in B-ALL, that these cells are found in all immunophenotypic populations, and that they show a high degree of malleability. We therefore believe that the stochastic CSC model is more appropriate to describe B-ALL.

Clonal Evolution and Tumour Heterogeneity

The picture of the CSC compartment in leukaemia is complicated by the process of clonal evolution, which can lead to significant genetic diversity among cancer-propagating cells. A recent study by Anderson et al. used a combination of fluorescently labelled probes to assess copy number alterations (CNAs) in selected genes in *ETV6/RUNX1*-positive ALL.[33] In the majority of cases, they observed substantial heterogeneity among propagating populations, with genetically distinct subclones being related to each other in ancestral trees rather than in a simple linear evolution model. In some cases, CNAs in the same gene appeared to arise independently. This genetic diversity was generally maintained following transplantation into NOD/SCID/IL2Rγ^{null} mice, with the authors again observing leukaemic regenerative potential in populations with multiple immunophenotypes. In many cases, different clones had different regenerative potency, with clones present at low frequency in initial samples sometimes becoming dominant in xenografts.

This work has been supported by a study from John Dick's laboratory, which compared CNAs in diagnostic *BCR/ABL1*-positive ALL samples with those xenografted into immunodeficient mice.[34] Samples from some patients showed the same CNA distribution in both the xenografts and diagnostic clones, while other xenografts showed genetic changes, with minor diagnostic subclones becoming dominant. Analysis of CNA alterations in samples where xenografts were repopulated by different subclones showed that CNAs were acquired at different stages of disease progression. As with the findings of Anderson et al., the subclones were related to each other through multiple branching rather than simple linear evolution.

These evolutionary trees suggest that a Darwinian-style selection process is occurring among leukaemia-initiating populations, with clones exhibiting increased tumourigenicity becoming increasingly dominant. It has also been shown that deletions of the *BTG1* gene can occur independently in different B-ALL subclones, providing further support for multiclonal evolution of ALL.[35] Several other groups have shown that relapse clones have most likely evolved from clones present at diagnosis, with the selective pressures of therapy (bottleneck) driving a Darwinian evolutionary process leading to resistant clones.[36–38] A recent study also demonstrated that clonal evolution and selection occur in xenografted human T-ALL samples, with the xenograft leukaemias exhibiting a more aggressive phenotype and genetic lesions resembling patient relapse samples.[39] This suggests that mouse xenograft models are highly relevant for studying clonal selection processes in relapse patients.

Taken together, these studies demonstrate significant genetic diversity within leukaemia-propagating populations. This process of clonal evolution and selection adds an additional layer of complexity, which must be incorporated into CSC models. These data show that there is significant genetic variation among cells with self-renewing properties, but when taken alone do not imply that such properties are common to every tumour cell. As such, the process of clonal evolution itself does not necessarily distinguish between the hierarchical and stochastic CSC models, as the Darwinian-style selection process would be expected to occur within a hierarchical CSC compartment. However, in combination with the data showing a high diversity and frequency of tumour-initiating cells, the high level of clonal diversity is consistent with the stochastic model for ALL and provides a mechanism by which a heterogeneous population of tumour-initiating cells can be generated. Clonal evolution also suggests that the propagating cell population evolves significantly through the tumour development process, meaning that their phenotypic properties may also vary at different stages of disease. This could help explain some of the current controversies over the identity and frequency of CSC populations. The limited number of CNAs analysed in the work described in this section means that these methods will provide a significant underestimate of the true clonal complexity of the ALL samples. A strategy to uniquely and heritably mark individual leukaemia cells, for example using cellular barcoding,[40] would provide a more comprehensive picture of the stem cell frequency, clonal complexity and evolution of ALL.

Cell of Origin

Another factor that may affect tumour evolution is the cell from which the tumour originates – a distinct concept to that of the CSC. The cell of origin refers to the normal cell that acquires the first oncogenic mutations, while the CSC refers to cells that propagate the tumour, so these cells may be phenotypically different.

Normal stem cells have often been suggested as the cell of origin, partly because they remain in tissues long enough to accumulate the mutations that drive oncogenic transformation. There is evidence that mutations in stem cells specifically drive cancer in some models. For example, the targeted deletion of adenomatous polyposis coli (APC, a negative regulator of the Wnt pathway) in mouse intestinal crypt stem cells results in transformation and adenoma growth, while APC deletion in transit-amplifying cells does not.[41] There is also evidence that HSCs are involved in the pathogenesis of some leukaemias, including chronic myeloid and lymphoblastic leukaemias.[42,43] However, the CSC hypothesis does not preclude the possibility that transformation occurs in more differentiated cell types, which then re-initiate stemness pathways. Lymphoid cells retain the ability to clonally expand even after maturation, so could be considered as unipotent stem cells. This means that reactivation of self-renewal programmes may be unnecessary in lymphoid tumours. Indeed, it has been demonstrated that memory T and B cells share aspects of a self-renewal programme with long-term HSCs.[44] The apparent plasticity and heterogeneity of ALL cells makes it difficult to precisely identify the cell of origin, as the transformed population may present different phenotypic features to the cell in which the initiating events occurred. For example, in mice, peripheral B cells can de-differentiate to early progenitors following loss of a single gene, *Pax5*, demonstrating a high degree of plasticity in B cell lineages.[45] It is therefore conceivable in principle that tumours that present with an immature B-cell phenotype originated in a more differentiated B cell. A high level of plasticity among leukaemia-initiating cells is further demonstrated by the case of *CALM/AF10* AML, where it appears that the disease is propagated by an LSC with lymphoid characteristics.[46] There are also examples of phenotype switches from ALL at diagnosis to AML at relapse (or vice versa),[47–49] suggesting a primitive cell of origin. An important study from Jacobsen's laboratory established that the *TEL/AML1* fusion is exclusively found in pro-B cells, but not multipotent progenitors, suggesting a cell of origin in more committed B-cell progenitors.[50] In contrast, P210 *BCR/ABL1* was found in multipotent HSCs, suggesting that the cell of origin varies depending on the initiating translocation.

An illuminating insight into the cell of origin in human leukaemia has come from a study by Barabé et al., which involved transducing primitive

human cord blood cells with *MLL* fusion genes and transplanting the cells into mice.[51] The authors studied immunoglobulin heavy chain (IgH) rearrangements, which occur early in B-lymphoid differentiation. In approximately 40 % of cases, the majority of IgH configurations were from the germline, indicating that transformation was occurring in a primitive cell type that had not yet initiated IgH rearrangement. In secondary recipients, the frequency of the germline IgH declined, indicating that the leukaemia-initiating cells were evolving from a primitive cell type to a more mature one with IgH rearrangements. This study again demonstrates clonal evolution and selection among leukaemia-initiating populations and confirms that the cell of origin is not identical to the leukaemia-propagating cells at later disease stages.

Another study, from Tariq Enver's group, assessed the cell of origin in *TEL/AML1* childhood ALL by examining IgH rearrangements in monochorionic twins with and without leukaemia.[17] Combined with a model of the disease involving transducing human cord blood cells with *TEL/AML1*, they established the presence of a pre-leukaemic clone that developed in utero. This clone was present in both twins, had a specific immunophenotype (CD34+CD38^{low}CD19+) and had self-renewal potential. In the leukaemic twin, the tumour-propagating cells were descended from this population and exhibited further rearrangements. These results suggest that the *TEL/AML1* translocation is able to generate a population of pre-leukaemic cells with stem cell-like features, which then acquire extra genetic changes to develop into leukaemia. This is also indicative of a hierarchy in the early stages of tumour development, although the standard NOD/SCID mice used may not efficiently engraft all potential leukaemia-initiating populations. Together, both studies show that initiating ALL mutations can occur in primitive cells, which then undergo a clonal evolution process leading to the formation of a developed leukaemia-propagating population.

Further understanding of the cell of origin has come from the development of mouse models of cancer, in which oncogenic events can be targeted to specific cell types to assess whether they can undergo transformation. McCormack et al. used lineage-tracing studies to show that thymic turnover in a transgenic murine T-ALL model was mediated by thymocytes expressing the *Lmo2* oncogene.[52] Normal thymic turnover is driven by bone marrow progenitors and not thymocytes, so this result indicates that the transgenic thymocytes gained stem cell properties and were most likely the cells of origin in this model. A recent study from Dupuy's laboratory demonstrated that the cell of origin in a T-ALL mouse model can affect the driver mutations which become selected in the resultant tumour evolution process.[53] They developed a Sleeping Beauty transposon model, in which transposase expression is under the control of an inducible Cre-based system, allowing initiation of transposon-mediated mutagenesis in different cells along the T-cell lineage. The results showed that altering the cell of origin led to varying subtypes of T-ALL, with altered genetic selection. In particular, HSC-derived tumours showed significant differences compared with those derived from more differentiated cells, notably a high prevalence of Notch1 mutations. HSC-derived tumours were also genetically more homogeneous and had fewer driver mutations. This suggests that more mutations may be required to transform differentiated cells than stem cells. Although this does not appear to concur with the idea that self-renewal can still occur in more mature lymphoid cells, it is possible that different types of lymphoid self-renewal favour different oncogenes. Overall, this study advances the possibility that differences in the cell of origin may be linked to the genetic heterogeneity observed in ALL.

In summary, the studies described above suggest various possible cells of origin in different ALL models. The process of clonal evolution among initiating cells means that the cell of origin is not likely to be phenotypically identical to the cells that propagate the tumour, so differences in the cell of origin do not differentiate between the hierarchical and stochastic CSC models. Recent findings suggest there are two distinct populations with LSC activity in many types of AML, with a more immature population giving rise to a mature one in a hierarchical manner.[4] This suggests that AML is a progenitor cell disease, and the stem cell hierarchy in AML may be comparable to normal haematopoiesis. In ALL, the fact that mature lymphoid cells can clonally expand means that many potential target cells for initiating mutations already have a degree of self-renewal potential, and can be considered as unipotent stem cells. If the development of ALL mirrors that of normal lymphoid cells, it follows from this that large numbers of transformed ALL blasts should also have self-renewal potential. This would agree with our results demonstrating a high frequency and diversity of ALL-propagating cells in xenograft models.

Clinical Relevance and Conclusions

In summary, there remains a significant amount of debate over the applicability of CSC hypotheses to ALL. The most recent data, using mouse models with greater immunodeficiency, suggests that tumour-propagating capacity is present in B-ALL blasts at a much greater frequency than was initially thought, and that these propagating cells have diverse immunophenotypes. This, combined with the increasing evidence that tumour cells undergo clonal evolution, resulting in genetically and functionally diverse ALL subclones, casts doubt on the idea of a tumour hierarchy containing a phenotypically distinct subset of CSCs and instead supports a more stochastic model for ALL. A more developed understanding of this issue is likely to require further improvement and standardisation of murine xenotransplantation models of human disease, along with the use of methods that allow *in vivo* lineage tracing of individual tumour cells to assess their contributions to tumour development. It has long been established that bone marrow from relapse patients has increased ability to proliferate in SCID mice,[54] while more recent work has shown a correlation between ALL engraftment time in NOD/SCID mice and high risk of early relapse.[55] Together with the study showing that clonal evolution in xenograft mouse models is analogous to that in relapse,[39] these results show that using ALL xenograft models to investigate leukaemic stem cell frequencies are highly relevant to the study of patient disease.

An understanding of the biology of ALL-propagating cells, and their differences with both normal cells and any tumour cells that lack this capability, is a crucial step towards the development of therapies that specifically target CSCs. However, the apparent genetic diversity of tumour-propagating cells in ALL means that therapies directed towards specific mutations may only be relevant in specific subpopulations. It is therefore important to identify key driver mutations, as opposed to secondary passenger mutations, which are not essential for the survival and proliferation of the leukaemia and may only occur in a subset of cells. It is also worth considering the phenomenon of oncogene addiction, where tumour cells rely heavily on certain oncogenic mutations for survival. This has been shown for other leukaemias, notably in CML where targeting the BCR/ABL fusion protein eradicates the bulk of the leukaemia,[56] raising the possibility that targeting ALL fusion genes could have similar effects. However, recent work has shown that BCR/ABL function is not essential for the

survival of CML stem cells,[57] suggesting that addiction to the initiating oncogene may be different in stem cells compared with the bulk of the population. This finding again highlights the importance of understanding the underlying biology of the propagating population to allow complete eradication of the disease.

A recent study has identified HSC- and LSC-specific gene signatures in AML cell populations that display stem cell activity in xenograft assays.[58] The presence of these signatures was a significant determinant of patient survival, providing the first indication that CSCs have relevance to clinical outcome. Another group has shown enrichment of this LSC signature, as well as a normal HSC signature, in early T-cell precursor ALL (ETP-ALL) samples.[59] ETP-ALL is an aggressive malignancy with poor outcome, so these results suggest that the enriched stem cell signature may be relevant in determining survival in some types of ALL. However, our results suggest there is a poor correlation between this gene signature and B-ALL blasts, and that the HSC signature is unable to distinguish between CD34^{high} and CD34^{low} blast populations.[27] This is not entirely surprising given the high frequency of leukaemia-initiating blasts compared with AML, and suggests that HSC gene signatures may have less clinical relevance in ALL.

As an alternative, the fact that mature lymphoid cells can clonally expand means they can be considered as unipotent stem cells. The high frequency of tumourigenic blasts in ALL suggests this concept can be applied to malignant as well as normal lymphoid development, meaning that ALL blasts may use a lymphoid self-renewal programme that is distinct from that of normal HSCs. The fact that we do not see clustering of ALL blasts and normal HSCs following analysis of the haematopoietic self-renewal signature supports this hypothesis. It follows from this that future therapeutics could potentially exploit differences between lymphoid and HSC self-renewal programmes. An improved understanding of the unique features that regulate clonal expansion in normal and malignant lymphoid cells is therefore a vital objective for the future. ■

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