Cancer stem cells in haematological malignancies

At least several types of human haematological malignancies can now be seen as 'stem-cell diseases'. The best-studied in this context is acute myeloid leukaemia (AML). It has been shown that these diseases are driven by a pool of 'leukaemia stem cells (LSC)', which remain in the quiescent state, have the capacity to survive and self-renew, and are responsible for the recurrence of cancer after classical chemotherapy. It has been understood that LSC must be eliminated in order to cure patients suffering from haematological cancers. Recent advances in LSC research have allowed for description of LSC phenotype and identification of potential targets for anti-LSC therapies. This concise review summarises the current view on LSC biology and targeted approaches against LSC.

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Introduction

Since the first application of nitrogen mustard derivatives to treat haematological malignancies in the 1940s, for decades the general idea of anti-cancer therapies was to eradicate as high a percentage of tumour cells as possible by applying the maximum tolerated dose of a toxic agent. Such agents were mainly designed to eliminate rapidly dividing cells as they were thought to be the most prominent feature of cancer in general. One of the immediate observations was, however, that even a vast reduction of cancer content in the patient's organism can be transient and subsequent recurrence can occur. This, along with parallel observations of robust heterogeneity among cancer cells, led to the idea of the existence of a distinct pool of 'cancer-initiating cells', which do not divide rapidly and thus are relatively resistant to classical chemotherapeutics, and which possess the ability to reconstitute the cancer bulk after therapy. That idea has deeply changed the understanding of therapeutic needs in cancer. This review briefly summarises the current state-of-the-art understanding of the role of cancer-initiating cells in haematological cancers. Unless otherwise stated, most of the information reviewed in this work relates to acute myeloid leukaemia (AML), as the best-studied disease in this context.

Stem cell paradigm in haematopoietic malignancies

Intratumoural heterogeneity spotted in most of the malignant cancers has puzzled researchers for decades. Two main models have been proposed to explain this phenomenon: the 'stochastic model' and the 'stem cell model'.

Stochastic model

The stochastic model is historically older [1]. Most of the cancer cells would have the same potential to self-renew and divide, and the choice between self-renewal and differentiation would be more or less random unless one of them is induced by external factors. Under this model, practically each cancer cell could reproduce the whole cancer. Also under this model, cancer existence would be mostly dependent on symmetric divisions of cancer cells, each of them giving rise to identical progeny. Cancer growth would then be highly disorganised, and only a small, random number of cancer cells would be arrested in the G0 phase of the cell cycle, and these cells would be responsible for the regrowth of cancer after chemotherapy.

Stem cell model

Opposing the stochastic model, the stem cell theory was proposed in the 1940s [2] and experimentally validated later on [3]. This concept assumes that cancers, especially haematological malignancies, resemble the hierarchical organisation of healthy haematopoiesis. In this model, a distinct set of stem cells would undergo a slow, asymmetrical division to produce an identical stem cell (self-renewal) and at the same time to produce a more differentiated progenitor cell. The progenitors would then undergo rapid

divisions to generate most of the cancer bulk within the given patient.

Multiple laboratories devoted their efforts to identify, describe, and target cancer stem cells. A great deal of progress has recently been made, especially in the field of haematological malignancies. However, as usual for cancer-related studies, the image generated by this research appears to be far more complicated than a simplistic assumption of one stem cell pool responsible for cancer regeneration. The landscape of 'stemness' in haematological malignant disorders is characterised by considerable complexity and dynamics, and is still a matter of intensive debate.

Given the fact that cancer can be mostly seen as a disease of the genes, the stem sell model raises the question of whether a sequence of gene alterations occurs in a healthy haematopoietic stem cell or, alternatively, a highly committed progenitor de-differentiates into stem-like tumour-initiating cell. Currently, while the occurrence of the latter situation cannot be excluded in some cases, more evidence has been gathered in support to the first option. That especially holds true for AML and myelodysplastic syndromes (MDS). Under this model, the process of leukaemia development (leukaemogenesis) would rely on a series of stepwise mutations within the primarily immature, multipotent, and quiescent haematopoietic stem or early progenitor cell (HSC). At some point, these transforming events accumulate to give rise to a 'leukaemia-cell of origin' (LCO), which is still considered to be a 'preleukaemic stem cell' (pre-LSC) [4]. If such cells are xenotransplanted to an immunocompromised host, they would produce leukaemia with variable penetration and considerably long latency. These cells need further genetic and/or epigenetic events to develop into a 'leukaemic stem cell' (LSC), capable of producing fully penetrant leukaemic growth with short latency in the xenotransplantation models.

It is important to understand the functional alterations harboured by LSC in comparison with the healthy HSC. The most obvious of these are reduced differentiation, decreased apoptosis, and deep metabolic changes. Also crucial is the alteration in the self-renewal and proliferative capabilities; while normal HSC divide mostly in an asymmetric manner, in LSC the chance for symmetric division producing two identical stem cells is greatly increased, as shown by mathematical modelling [5].

Heterogeneity of leukaemia stem cells

A great deal of evidence has been gathered in support of the hierarchical organisation of the leukaemic growth, resembling to some extent the natural haematopoiesis process. What must be remembered, however, is that LSC are characterised by dramatically increased genetic instability. This translates into an increased chance of random events within their genome and, during subsequent symmetrical divisions, would eventually translate into generation of a number of LSC subclones within a given cancer. All surviving LSC subclones would retain their self-renewal and leukaemia-initiating capabilities, but may gain new oncogenic drivers and differentially respond to the therapeutic approaches. If true, this model has profound impli-

cations for targeted therapies in leukaemia and explains why 'narrow-spectrum' targeted therapies have a considerably high chance of failure in the long term, even if they are designed to disturb functioning of LSC. It would also explain difficulties in unequivocal description of the molecular phenotype of the 'universal' LSC.

Methods of identification of haematopoietic cancer stem cells

Xenotransplant models

Identification of the LSC via xenotransplant-based models relies on the determination of whether a given cell type stably reproduces all populations of the original malignancy in the recipient animal. The first evidence, based on the xenotransplantation models, for the functioning of LSC in human haematopoietic malignancies has been provided in the 1990s [6, 7]. The main limitation for this assay is the fact that it depends on the ability of studied cells to home to the mouse bone marrow, which does not necessarily have to be a feature of a human stem cell. The other issue is related to differences between humans and mice regarding the microenvironmental conditions within the stem cell niches in bone marrow. Nevertheless, the limiting dilution analysis (LDA) via transplantation to severe combined immunodeficient (SCID), nonobese diabetic (NOD)/SCID, or, recently, NOD/SCID/IL2rγnull (NSG) mice has become one of the standard methods for functional identification of LSC. The LSC frequency identified by these techniques varies and is estimated from approximately 1 in 1.6×10^3 to 1 in 1.1×10^6 cells, and was shown to be usually highest in the CD34+CD38– fraction [8]. Conspicuously, higher frequencies of stem cells in acute myeloid leukaemia were shown to correlate with robust minimal residual disease and poorer survival of patients [9].

Cell surface markers

While molecular phenotype of LSC relates to multiple features in various cellular compartments, in the era of flow cytometry-based cell sorting the utilization of cell surface markers provides a high-level convenience for isolation of LSC. Initial studies were focused on the CD34+CD38–Lin– population of leukemic cells [7]. Further on, LSC phenotype was shown far more complex and numerous other markers were proposed useful in LSC phenotyping. These include CD71, CD90, CD117, CD123, HLA-DR [10], or CD33 [11] for AML, CD26 for chronic myeloid leukaemia (CML) [12], or CD133 for acute lymphoblastic leukaemia (ALL) [13].

From a therapeutic point of view, it is important to identify the cell surface markers that can distinguish between normal HSC and LSC. Normal HSC are usually of the following phenotype: CD34+/CD38–/CD13–/CD33+/CD90+/CD123–lo/CD117+/CD71+ [14]. Aberrant surface phenotype of AML stem cells usually lacks the expression of CD90, CD117, and HLA-DR but is robust for CD123, CD96, CD44, C-type lectin-like molecule-1 (CLL-1) [15], or CD47 [16]. There is some debate on the expression of CD33 molecule on AML LSC [11] and its importance for anti-AML therapies. Interestingly, unlike regular HSC, the LSC can sometimes

be devoid of CD34 expression [17]. New markers are also being proposed based on proteomic analyses [18].

In vitro functional assays

As the xenotransplantation-based methods are time- and effort-consuming and may raise bioethical issues, several *in vitro* methods have been proposed in order to identify stem-like properties of leukemic cells. Some of the most often used are: aldehyde dehydrogenase (ALDH) activity and Hoechst 33342 efflux assay [19] or divisional kinetics using PKH staining [20]. None of these methods seems currently superior in its precision to the limited-dilution transplantation, but each of them can serve as a rapid screening technique for new candidates for stem cells in a number of malignancies, not necessarily of haematological origin.

As the aforementioned *in vitro* functional assays rely on the natural properties of stem cells in general, they might useful for distinguishing between HSC and LSC only if combined with other methods, e.g. immunophenotyping. This is best seen in the case of ALDH activity, where normal haematopoietic stem cells within the bone marrow of AML patients have been shown to present usually a higher ALDH activity level than co-existing leukaemic stem cells [17].

Genetic profile of leukaemia stem cells

Following the introduction of microarray technology and subsequent modern next-generation sequencing (NGS) methods (reviewed in [21]), much effort has been devoted to identify genomic [22], transcriptomic [23, 24], and epigenetic [25] signatures of LSC (reviewed in [26]). The importance of these approaches has been underscored by the finding that stem cell gene expression programs have a profound effect on the clinical outcome in human leukaemia [8].

During sequencing of the AML genome, for instance, two distinct classes of somatic genetic alterations were recognised, among other variations. The first group is related mostly to the increased proliferative capabilities of LSC, and includes such genes as *FLT3, Ras*, or *c-Kit*. The second set of mutations contains alterations that induce maturation block, such as changes in the sequence of *CEBPA, CBFB-MYH11, NPM1, DEK-NUP214, PML-RARA, MLL-MLLT3*, or *RUNX1-RUNX1T1* [27]. Additionally, deviations in the sequences of epigenetic modifiers (such as *IDH1, IDH2*, and *DNMT3A*) have been identified [28]. The image emerging from these experiments supports the 'two-hit' hypothesis of LSC formation. However, genetic studies also show that LSCs display a high degree of genetic variety [29], which reduces the chances for prospective development of a single universal anti-LSC treatment modality.

An interesting observation provided by next-generation techniques is that age-related changes in HSC, e.g. within the *TET2* gene, contribute strongly to the pathogenesis of monoclonal B cell lymphocytosis (MBL) and chronic lymphocytic leukaemia (CLL) [30]. This raises hope for potential development of early screening methods capable of identifying patients with increased risk of development of mature lymphoid malignancy or perhaps the therapeutic prevention strategies against these disorders.

Biological and molecular basis for 'stemness' in haematopoietic malignancies

LSC Niche

Normal HSC residing in bone marrow (BM) depend heavily on support from specialised and strictly organised stem-cell niches, endosteal and vascular/peri-sinusoidal. The dialogue with other components of the niche is crucial for HSC self-renewal, survival, and functioning. Multiple extracellular and intracellular factors contribute to this phenomenon (reviewed in [31]), including haematopoietic growth factors (such as interleukin (IL)-3, IL-6, Flt3-ligand, LIF, TPO, SCF) and their cognate receptors, signalling pathways (e.g. Wnt- or Notch-mediated), molecules regulating cell cycle (e.g. p53), and numerous transcription factors or regulators (such as HOXB4, SCF/TEL-1, RUNX1, STAT5 [32]). Recently collected evidence suggests that LSCs robustly benefit from the support of bone marrow niches [33]. Leukaemia stem cells hijack the BM niche structures for the needs of their leukaemic growth and thus suppress the normal haematopoiesis. Thanks to their interactions with the endosteal niche, LSC can maintain their dormant state and are protected from the cytotoxicity of classical chemotherapy [34]. Leukaemia stem cells are retained in the BM niche primarily via interactions of CXCL12 chemokine (also known as stromal cell derived factor-1 – SDF1) with its cognate receptor CXCR4 [35], if it is expressed on LSC. These interactions are responsible for upregulation of the expression of adhesion molecules VCAM1 and VLA4. Some role has been also proposed for CD44, acting as an adhesion molecule and a receptor for osteopontin within the BM niche (reviewed in [36]).

Self-renewal pathways

As mentioned above, the aid provided by the microenvironment of the BM niche induces within LSC signals transduced via the intracellular pathways responsible for self-renewal of the stem cells (reviewed in [37]). These include phosphoinositide-3-kinase (PI3K)-, homeobox (HOX)-, Notch-, Hedgehog-, and Wnt/β-catenin-mediated cellular signalling. Also important for LSC quiescence are the signals transduced via transforming growth factor β (TGF-β) superfamily signalling and molecules of the SMAD family [37].

Cell survival and proliferation pathways

Aberrant, sustained activity of two major signalling pathways, nuclear factor (NF)-κB [38] and PI3K/Akt/mammalian target of rapamycin (mTOR) [39], in orchestration with a set of antiapoptotic molecules (e.g. Bcl-x and Bcl-2 [40]), are held responsible for the uncontrolled growth and decreased apoptosis in LSC.

Therapeutic targeting of haematopoietic cancer stem cells

Increasingly better phenotyping of LSC along with the growing understanding of the events leading to transformation from normal HSC to a malignant LSC, and then self-renewal and survival of LSC, have led to identification

of a range of potential therapeutic targets in haematopoietic cancers [41].

Before going into more detail, it is crucial to understand two important aspects of therapeutic targeting against LSC. First, the above-mentioned signalling pathways create a dense, overlapping, and cross-talking network of molecular events within LSC. That translates into the fact that targeting a single event/pathway within LSC would, in many cases, result in only transient success. It is necessary to use the personalised medicine approach to clearly describe the aberrant networking within LSC of a given patient in order to tailor the best-fit set of targeted therapies in the particular case. Second, monitoring of the immediate outcome of anti-LSC therapies is far more complex than a standard classification into no response, partial response, or complete response etc., usually applied to describe the effectiveness of chemotherapy. It has become clear that assessment of anti-LSC effectiveness requires more sophisticated and precise methods [42].

Strategies for targeting LSC can be divided in two manners. The first division takes into consideration the chemical properties of the therapeutic agent. Here, two major classes emerge: small molecules (e.g. targeting the kinase activity of an oncogene or blocking molecular interactions) or antibody-/protein fusion-based drugs (targeting mostly surface molecules or extracellular growth factors). The second type of classification relates to the type of target. Here, we distinguish agents targeting adhesion molecules, growth factors and their receptors, intracellular signalling molecules, antiapoptotic molecules, or transcription factors.

Multiple targeted strategies have been attempted experimentally since the first identification of LSC. Only some of them have reached the clinical trial stage. The most prominent success has been achieved with BCR-ABL oncoprotein inhibitors in CML (although their effectiveness against LSC seems to be only partial [see below]), and many attempts are currently being undertaken to reproduce this success in other haematological malignancies. The examples applied in leukaemia treatment are presented in Table 1.

What must be remembered, again, is that even in the case of clearly identified oncogenic lesion, like BCR-ABL fusion protein in CML, the LSC can still develop overlapping pathways to ensure survival under unfavourable conditions, as is the case in CML. Recent data suggest that CML LSCs are relatively resistant to BCR-ABL kinase-targeting drugs [43] and may require the addition of e.g. PI3K-[44] or mTOR-targeted [45] strategies in order to achieve high level of LSC eradication. That underscores the need for systems biology-based analysis of each case and the combinatory, personalised approach to the targeted treatment of each patient suffering from haematological malignancy. It is also worth to mention the need for profound understanding of interactions of LSC with the patient's immune system in order to prospectively develop active immunisation strategies that can help eliminate minimal residual disease [46].

Future perspectives

When in 1971 Richard Nixon declared the "war on cancer", it was hoped that malignancies would be eradicated within the next 20/30 years. In 2014, cancer still remains a major cause of death and its frequency has dramatically increased in developed societies. However, researchers in this war have won many battles. The area of stem cell studies in haematological malignancies has become one of the most vivid and dynamic topics in current oncology. From what we have learned about cancer stem cells, great hope has emerged for the development of highly successful anticancer therapies that might eventually combat most cases of this deadly disease. Many challenges remain, with the need for undisputable identification and quantification of LSC in each type of malignancy and finding the right therapeutic combinations to minimise the chance of recurrence. Nevertheless, much evidence suggests that forty-three years after Richard Nixon's declaration, we are on the right track to eradicate haematological cancer via targeting its stem cells.

Table 1. Examples of potential anti-LSC targeted agents under investigation in leukaemia (based on [47])

Type of target/therapy	Targeted structure/pathway	Therapeutic agent(s)
Cell surface markers	CD33	Gemtuzumab ozogamicin CART33
	CD44	Monoclonal antibody
	CD47	Monoclonal antibody
	CD96	Monoclonal antibody
	CD123	Diphtheria toxin-IL-3 fusion protein Monoclonal antibody
Pathway-targeted therapies	Hedgehog signalling pathway	Sonidegib
	NF-κB signalling pathway	Parthenolide Dimethylaminoparthenolide Bortezomib
	c-Kit, Src tyrosine kinases	Dasatinib
Anti-apoptotic molecules	Bcl2	ABT-199 Oblimersen

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