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# Studying leukemia stem cell properties and vulnerabilities with human iPSCs

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## Abstract

The reprogramming of cancer cells into induced pluripotent stem cells (iPSCs) can capture entire cancer genomes, and thus create genetically faithful models of human cancers. By providing stringent genetically clonal conditions, iPSC modeling can also unveil non-genetic sources of cancer heterogeneity and provide a unique opportunity to study them separately from genetic sources, as we recently showed in an iPSC-based model of acute myeloid leukemia (AML). Genetically clonal iPSCs, derived from a patient with AML, reproduce, upon hematopoietic differentiation, phenotypic and functional heterogeneity with all the hallmarks of a leukemia stem cell (LSC) hierarchy. Here we discuss the lessons that can be learned about the LSC state, its plasticity, stability and genetic and epigenetic determinants from iPSC modeling. We also discuss the practical and translational implications of exploiting AML-iPSCs to prospectively isolate large numbers of iLSCs for large-scale experiments, such as screens, and for discovery of new therapeutic targets specific to AML LSCs.

## 1. The concept of AML-LSCs and its clinical relevance

### 1.1. Cancer stem cells

The cancer stem cell (CSC) model posits that cancers – or at least some of them – are hierarchically organized, with CSCs residing at the apex of this hierarchy. The concept and associated properties of CSCs were formulated as a response to the need to explain two important and nearly universal observations in cancer biology, tumor pathology and clinical reality. The first was that cancers are heterogeneous in many observable ways: histopathologically; in terms of expression of marker genes or cell surface markers; global



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NS wrote the manuscript. EPP conceived the content, wrote and edited the manuscript.

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gene expression; cellular and molecular phenotypes, such as proliferation and metabolism; and functional readouts, such as the potential to initiate cancer in xenograft models or metastasize. The second observation was that cancers frequently relapse. Thus, concurrently with studies documenting the existence of stem cells in various tissues (tissue-specific or adult stem cells), the idea that cancers are maintained by stem cells, the CSCs, that generate, maintain and can re-establish their heterogeneity, and from which relapse arises, emerged. Since then, the CSC model was refined and still applies to at least some cancers to this day, although the near-universality of this phenomenon has been challenged and more nuance has been overlaid upon the original definitions, in relation to tumor types, context dependencies and variation in frequency of cells with CSC properties (Meacham and Morrison, 2013; Nassar and Blanpain, 2016).

The CSC model endowed CSCs with properties of normal tissue-specific stem cells. Thus CSCs have two defining characteristics: (1) the ability to self-renew and thus re-grow and maintain the cancer; and (2) the ability to give rise to more differentiated cells that themselves cannot initiate or maintain the cancer, thus generating tumor heterogeneity. Following the paradigm of normal tissue-specific stem cells that give rise to a variety of progeny with limited capacity to proliferate, CSCs differentiate into cells that form the bulk of cancer tissue and have limited tumorigenic capacity themselves. This transition is thought to be mediated by epigenetic alterations and to be irreversible or at least rarely reversible.

### 1.2. Leukemia stem cells

AML LSCs are the prototypical CSCs, as AML was the first malignancy in which a stem cell population could be identified and prospectively isolated (Lapidot et al., 1994; Bonnet and Dick, 1997). AML LSCs share many properties with normal hematopoietic stem cells (HSCs), including self-renewal and multipotency and can initiate and maintain AML by giving rise to identical, as well as more differentiated, daughter cells that cannot propagate the disease. AML LSCs are classically defined by their functional properties in mouse or xenotransplantation models (Lapidot et al., 1994; Bonnet and Dick, 1997; Kreso and Dick, 2014). Seminal work by John Dick's group in the past two decades established the assays and principles that are now commonly held in this field. Human AML LSCs are typically studied in xenograft models, whereby cells obtained from patients are transplanted into immunodeficient mice, i.e. mice with a compromised immune system that allows them to receive human cell grafts without rejecting them. The definition and properties of LSCs are thus dictated by their observed properties in xenotransplantation assays: self-renewal ability, defined as the ability to give rise to leukemic engraftment that can be maintained over serial transplantation; and differentiation into more mature progeny that, themselves, cannot engraft leukemia (Lapidot et al., 1994; Bonnet and Dick, 1997; Kreso and Dick, 2014).

AML is a hematologic malignancy with a fulminant course and long-term overall survival rates of less than 30% for adults (Dohner et al., 2010). While most patients initially respond to high-dose chemotherapy, many subsequently relapse. Accumulating evidence suggests that patient outcomes, such as overall survival and minimal residual disease after chemotherapy, correlate with measures of LSC activity, such as engraftment in immunodeficient mice and LSC gene expression (van Rhenen et al., 2005; Ran et al., 2009;

Pearce et al., 2006; Eppert et al., 2011; Ng et al., 2016). LSC frequency and activity in AML have also been associated with chemotherapy resistance and probability of disease relapse (Pollyea and Jordan et al., 2017; Thomas and Majeti, 2017a). Thus, the existence and frequency of LSCs in AML is considered a major determinant of the disease course and response to treatment and therefore understanding the biological properties of LSCs and characterizing their molecular signatures holds promise for developing better therapies.

### 1.3. Limitations of current xenotransplantation models of AML LSCs

The gold-standard experimental approach to the study of LSCs (and CSCs more broadly) has two components. First, a cell fraction containing the CSCs is prospectively isolated, based on cell surface markers or, more frequently, combinations of markers, typically by fluorescence-activated cell sorting (FACS). This is then transplanted into immunodeficient mice. In the case of AML, leukemic engraftment, detected after a certain length of time in the bone marrow of the recipient mice, means that the transplanted cells contained LSCs, whereas absence of engraftment, and hence, leukemia initiation potential, indicates absence of LSC potential.

However, the very nature of these assays also imposes considerable constraints to the study of LSCs. The first important limitation is the lack of specific and universal cell surface markers to identify, isolate and study AML LSCs, which typically comprise a small fraction of the bulk AML cells. While typically AML LSCs have the CD34+/CD38− immunophenotype, similarly to normal HSCs (Lapidot et al., 1994; Bonnet and Dick, 1997), more recent studies established that LSCs are also contained in the CD34+/CD38+ and even in CD34− cell fractions, especially upon disease progression, while some types of AML are notably CD34− (for example those with NPM1c mutation) (Bonnet and Dick, 1997; Eppert et al., 2011; Ishikawa et al., 2007; Sarry et al., 2011; Ho et al., 2016; Goardon et al., 2011; Pabst et al., 2016; Taussig et al., 2010). As there is no universal marker or combination of markers that can isolate LSCs, markers alone cannot be relied upon to assess LSCs or other CSCs without being combined with xenotransplantation or another functional assay reading out tumor initiation and long-term maintenance. Thus, even though quite widespread, the practice of using marker combinations as surrogates for LSCs can lead to erroneous assumptions and results.

On the other hand, while xenotransplantation is the gold standard for the identification and study of LSCs, the need to functionally define LSCs as cells that reconstitute the disease in immunodeficient mice (Lapidot et al., 1994; Bonnet and Dick, 1997; Kreso and Dick, 2014) adds substantial cost, time and requirement for specialized expertise and is increasingly appreciated to suffer from important drawbacks. Principal amongst them is that engraftment ability has now been shown to be influenced by the host strain and the genetic and clonal composition of the cells, which can both be critical confounding factors to the qualitative and quantitative assessment of LSCs (Klco et al., 2014; Reinisch et al., 2015; Wunderlich et al., 2010). The first xenotransplantation assays used SCID (Lapidot et al., 1994) and later NOD-SCID mice (Bonnet and Dick, 1997). Since then more permissive mouse strains were developed, including the commonly used NOD/SCID/IL2RG$^{-/-}$ (NSG) and NOD/Rag/IL2RG$^{-/-}$ (NRG) mice. Additionally, humanized strains expressing human hematopoietic

cytokines, such as the NSG-SGM3 mice, constitutively expressing human SCF, GM-CSF and IL-3 (Wunderlich et al., 2010), the MSTGR mice, expressing human M-CSF, TPO, IL3 and GM-CSF (Saito et al., 2016; Rongvaux et al., 2014) and others were developed (Nicolini et al., 2004). Engraftment of patient AML cells and thus the reported frequency of LSCs in different mouse models is variable between studies and influenced by the level of immune deficiency of the host strain (Klco et al., 2014; Goyama et al., 2015). More recent xenograft models using subcutaneous ossicles providing a humanized microenvironment are showing yet higher levels of human AML engraftment (Reinisch et al., 2016). Thus, the choice of mouse model can significantly impact the detection and measured frequency of LSCs.

The LSC – and more generally the CSC – model postulates that cancers are hierarchically organized as a result of non-genetic determinants and that cells with LSC properties exist at a distinct epigenetic state within a genetically homogeneous cancer cell population. In recent years large-scale sequencing of AML genomes provided a much more detailed picture of the mutational landscape of AML and revealed the extent of genetic and clonal heterogeneity present among patients and within the same patient (Cancer Genome Atlas Research et al., 2013; Papaemmanuil et al., 2016). The genetic and clonal composition of a given AML was shown to be a strong determinant of engraftment potential (Klco et al., 2014) with different subclones exhibiting a high degree of variation in their engraftment potential, suggesting that distinct AML genotypes, clones and subclones may differ in their LSC activity and frequency. However the inter-clonal engraftment variation may also be selected by the mouse strain and not be an inherent property of the AML clones. In any case, genetic heterogeneity, which is almost invariably present in AML patient samples, is a significant confounder of LSC studies that is only infrequently accounted for.

Recent experimental evidence is casting some doubts on the clinical relevance of patient-derived xenograft (PDX) readouts, specifically on how well they correlate to patient outcomes. It appears that most xenografts do not recapitulate the clonal architecture of the primary AML and subclones giving rise to clinical relapse do not generally show superior engraftment potential (Klco et al., 2014). Furthermore, the selection forces driving genetic drifts and genetic evolution in PDX models, seem to be different than those acting in patients, leading to increased genomic divergence between the models and the primary leukemia over time (Hussein et al., 2011; Ben-David et al., 2018, 2017). The above collectively raise questions as to how well reading out LSC potential in xenograft assays can inform patient risk and prognosis.

Lastly, xenograft assays, being laborious and costly, are not amenable to high throughput. In vitro or ex vivo models could allow scaling up experiments to expand the capabilities of experimentation to, for example, enable functional genetics, drug testing or genetic and chemical screens. However, such models are practically non-existent. Dick and colleagues reported the derivation of an AML cell line from primary cells that recapitulates their hierarchical organization (Lechman et al., 2016). On the other hand, primary AML LSCs – sorted and retrospectively ascertained after xenotransplants –have very limited ability for ex vivo growth.

## 2. A new iPSC-based model of AML LSCs

We recently reported a new model of AML LSCs (Wesely et al., 2020; Kotini et al., 2017) (Fig. 1). We derived iPSC lines from a female patient with AML (AML patient 4) through transient ectopic expression of the four Yamanaka factors. The patient's AML genetics included deletion of chromosome 7q in the context of a complex translocation involving chromosomes 1, 7 and 14, and a subclonal *KRAS* G12D mutation. The process of reprogramming globally resets the epigenome but maintains the genome intact. Multiple iPSC lines could be derived that contained the genetic lesions of this specific AML, namely the translocation and chromosome 7q loss. Some of them additionally contained the *KRAS* G12D mutation (Kotini et al., 2017). The AML epigenome was largely erased by the reprogramming process and these AML-iPSCs were indistinguishable from normal human pluripotent stem cells (hPSCs) in terms of phenotype, transcriptome, chromatin and teratoma formation ability (Kotini et al., 2017; Chao et al., 2017).

Upon in vitro differentiation with a protocol yielding primarily definitive-type hematopoiesis through a hemogenic endothelium intermediate, leukemic features were reestablished in the hematopoietic stem/progenitor cells (HSPCs) derived from these AML-iPSC lines. Phenotypically, the AML-iPSC-derived hematopoietic cells exhibited block in their maturation and markedly enhanced proliferation and survival ability, such that CD34+ myeloid cells with immature morphology could be propagated for weeks in culture. Furthermore, these cells recapitulated molecular features of primary AML cells as revealed through transcriptome analyses. Even more remarkably, they were able to engraft NSG mice with high efficiency and serially transplant a lethal leukemia (Wesely et al., 2020; Kotini et al., 2017).

The reprogramming of AML cells generally presents challenges and therefore a very small number of AML-iPSC lines exist today (Kotini et al., 2017; Chao et al., 2017). In comparison to other existing AML-iPSCs generated by us and Chao et al., all lines derived from this patient (with and without the *KRAS* mutation) exhibited exceptional engraftment capability in NSG mice. This is especially remarkable as blood cells derived from hPSCs are notoriously unable to engraft (Wahlster and Daley, 2016). In fact, AML-iPSC-derived hematopoietic cells are the only example of hematopoietic cells derived from hPSCs through directed in vitro differentiation without ectopic expression of transgenes that can engraft.

Furthermore, we found that hematopoietic cells derived from these "exceptional engrafter" AML-iPSC lines exhibit phenotypic and functional heterogeneity and hierarchical organization, recapitulating an LSC hierarchy. These findings were prompted by the observation that upon differentiation these AML-iPSC lines produced a population of cells with adherent growth that formed a monolayer with cobblestone morphology. Such an adherent cell population is never present in differentiation cultures of iPSC-derived HSPCs. We then found that these adherent cells had the typical immunophenotype of hematopoietic stem cells: $CD34^{+}/CD38^{-}/CD90^{+}/CD45RA^{-}/CD49f^{+}$ and that they continuously generated in culture daughter cells that grew in suspension as typical hematopoietic cells. Separation of the two cell fractions – adherent and suspension – and replating; sorting and re-culture; and a series of other experiments also involving time-lapse imaging and serial transplantation,

showed that these cells exhibit the two cardinal properties of LSCs: ability to self-renew and serially engraft leukemia and to give rise to more differentiated cells. Through a combination of in vitro and in vivo assays we showed that this fraction, that can be readily isolated prospectively by means of adherent in vitro growth, which we called “iLSCs”, resides at the apex of a hierarchy that fulfilled the hallmark features of LSCs: leukemia-initiating activity in vivo in NSG mice, including upon serial transplantation (reading out the property of self-renewal) and re-establishment of heterogeneity in vitro. Several independent lines of inquiry supported the LSC nature of iLSCs. Through serial replating assays, fate tracking at the single-cell level and mathematical modeling approaches we established the presence of a hierarchy whereby iLSCs give rise to more differentiated cells (referred to as “iBlasts”).

We also showed that iLSCs have gene expression and chromatin accessibility signatures of primary human HSCs and LSCs (Eppert et al., 2011; Jaatinen et al., 2006; Gentles et al., 2010). Through integrated genomics analyses of bulk and single-cell transcriptome and chromatin accessibility data we generated a new LSC gene signature. Furthermore, through genome-wide integrative molecular analyses followed by functional validation, we found that iLSCs are dependent on the activity of the transcription factor (TF) RUNX1. We thus identified RUNX1 as a critical regulator sustaining the properties of LSCs and characterized the relevant RUNX1 target genes.

This new model of AML LSCs affords some unique capabilities. First, it enables easy prospective isolation of a cell fraction highly enriched for LSC activity, merely by means of adherence to plastic, without cell sorting. Second, unlike primary AML LSCs, iLSCs can undergo self-renewing divisions in vitro and expand for several days up to a few weeks in culture, reaching numbers of tens or hundreds of millions of cells. Additionally, since the iPSCs they are derived from can be grown indefinitely at the pluripotent state, effectively unlimited numbers of iLSCs can be obtained, enabling for the first time large-scale experiments, including small molecule screens. Our mathematical modeling together with the functional analyses showed that the cellular composition of the system (iBlast/iLSC ratio) is relatively stable over a window of several days following hematopoietic specification, which allows an extended window for experiments, affording some degree of flexibility in its use.

### 2.1. Lessons for LSC biology

iPSC modeling of human LSCs can aid the study of both LSC biological properties and LSC research towards translational goals.

**Is adhesion linked to the LSC state?—**One characteristic property of our iLSCs that first alerted us to the presence of heterogeneity in these cultures and prompted us to further investigate its origins, is their adherent growth in tissue culture-treated dishes. In contrast, their more differentiated progeny, the iBlasts, grew in suspension. Although the practical implications of this phenomenon can be harnessed to readily separate the two fractions by means of adherence, the biological implications of these adhesion properties are presently less clear.

Homing and adhesion to the bone marrow (BM) microenvironment niches can be an LSC property beneficial for leukemia initiation and establishment (Konopleva and Jordan, 2011). Links between expression of several adhesion molecules to patient outcomes and LSC activity have been reported and their modulation explored as a potential therapeutic avenue (Rombouts et al., 2004). These include integrins, such as VLA-4 and CD44, selectins, and the CXCL12 (SDF-1)/CXCR4 chemokine receptor axis, which may also cross-talk with integrins. A monoclonal antibody against CD44 was shown to eradicate AML LSCs in xenograft mice (Jin et al., 2006). Neutralizing CXCR4 antibodies prevented both homing and leukemia initiation of primary human AML cells, as well as their long-term maintenance in xenograft recipients, while the same treatment did not affect the engraftment of normal HSCs (Tavor et al., 2004). Inhibiting adhesion mediated by VLA-4 and CXCR-4 has been proposed as a means to overcome chemotherapy resistance and relapse by mobilizing LSCs out of the BM niches (Matsunaga et al., 2003, 2008; Zeng et al., 2006, 2009; Fierro et al., 2009).

While in vivo homing and adhesion to BM niches involves complex interactions, adherence to regular tissue culture-treated dishes in culture is primarily mediated by integrins binding to extracellular matrix, while selectins and other adhesion molecules are typically involved in cell–cell interactions. We found that enforced detachment to prevent adherent growth did not affect iLSC properties, such as immunophenotype and engraftment potential, in our study (Wesely et al., 2020). Furthermore, iLSCs and iBlasts had comparable in vivo homing ability and other AML-iPSC-derived LSCs do not possess enhanced adherence properties in tissue culture (Chao et al., 2017). These observations, taken together, do not support a direct link between homing, adhesion and LSC function. It is still, however, possible that common regulators of adhesion and the LSC state play a role in endowing cells with LSC activity.

**Is the LSC state reversible?—**To thoroughly characterize the hierarchical relationships within the iLSC cultures, we employed a combination of mixed culture and sort-and-reculture experiments; mathematical modeling; single-cell plating and fate-tracking; and time-lapse imaging experiments (Wesely et al., 2020). These showed that, even though the hierarchical relationship was overwhelmingly in the direction of iLSCs (adherent cells) giving rise to iBlasts (suspension cells), a low-rate reversion of iBlasts to iLSCs could not be excluded. While it is possible that imperfect separation of iBlasts and iLSCs may confound this conclusion and, also, importantly, our calculations are constrained by the assumptions of the Markovian mathematical modeling approach we used, there are precedents for a bidirectional relationship between solid tumor CSCs and their more mature derivatives (Meacham and Morrison, 2013; Gupta et al., 2019).

Conversion of transformed human mammary epithelial cells into CSC-like cells has been described, albeit in a tissue in which non-malignant differentiated cells can spontaneously dedifferentiate into stem-like cells (Chaffer et al., 2011; Gupta et al., 2011). Additional examples of acquisition of stem cell-like properties and de novo generation of CSC-like cells from more differentiated non-CSCs in other epithelial tissues, sometimes in response to external stimuli, exist, mostly in mouse models (Schwitalla et al., 2013). Although such bidirectional conversion has not been documented in AML or normal hematopoiesis, the classic hematopoietic hierarchy is increasingly now found to be less rigid than previously

thought (Eaves, 2015). It is possible that the LSC state is more plastic and dynamic than previously appreciated and that this may furthermore be influenced by cell-intrinsic factors, stochasticity, microenvironmental factors, as well as drug exposures.

**Will all AML-iPSCs exhibit an LSC population?—**Another important question that remains unanswered is the generalizability of this iLSC population in other AML-iPSC lines derived from other patients and other AML genotypes. The lines we analyzed were all derived from one AML patient harboring a complex translocation resulting in loss of chromosome 7q (Kotini et al., 2017). The same iLSC population was observed in multiple iPSC lines derived from this patient, including iPSC lines derived from a subclone of the patient's leukemia, harboring an additional *KRAS* G12D mutation, in addition to the translocation (Kotini et al., 2017). These lines exhibited exceptionally high engraftment potential in NSG mice, serially transplanted a lethal leukemia and could be propagated long-term in vitro as immature CD34+ cells (Kotini et al., 2017). We have not observed this combination of properties in any of the few currently existing AML-iPSC lines (Kotini et al., 2017; Chao et al., 2017). Thus, it is currently unclear whether an in vitro self-renewing LSC/CSC population will be found upon differentiation of other AML-iPSC lines and iPSCs from other types of cancers and whether, if it is found, it will similarly exhibit high in vitro adhesion properties.

Because reprogramming of AML cells and primary cancer cells from solid tumors is technically challenging (Papapetrou, 2016a, 2019a, 2019b), very few such iPSC lines currently exist, and several more tumor cells will need to be reprogrammed in the future before this question can be answered conclusively.

**What is the impact of reprogramming on the iLSC state?—**A very interesting question that is related to the question above is whether the iLSC properties that we observed are impacted or even endowed by the process of reprogramming itself. Indeed, iLSCs appear to be even more potent in terms of leukemia initiating ability and in vitro self-renewal than primary AML LSCs. Reprogramming to a pluripotent state causes a massive resetting of the chromatin and epigenetic properties of the starting cell to the extent that it is effectively a "naked" genome of the starting cell that is captured in an iPSC, which has now become largely agnostic to its cellular origins (Papapetrou, 2016a, 2016b). We and others showed that AML cells reprogrammed into iPSCs exhibit no overt cellular or molecular malignant features at the pluripotent state (Kotini et al., 2017; Chao et al., 2017). It is only upon specification of the hematopoietic lineage that leukemic properties are re-established (Kotini et al., 2017; Chao et al., 2017). This constitutes clear evidence that, first, a specific cellular milieu is necessary for LSC properties to manifest and, second, that the AML genome alone is sufficient to re-establish leukemic properties upon exposure to the appropriate cellular environment. The processes of reprogramming and differentiation are both accompanied by dramatic genome-wide changes in accessible chromatin, histone marks and chromatin architecture (Papp and Plath, 2013; Apostolou and Hochedlinger, 2013; Hochedlinger and Jaenisch, 2015) and are, essentially, processes moving in opposite directions, with reprogramming erasing somatic cell epigenetic characteristics and in vitro directed differentiation establishing hematopoietic cell fate-specific chromatin marks and

gene expression programs. Reprogramming to pluripotency can reverse features associated with cellular aging, such as telomere length, mitochondrial and nuclear morphology, heterochromatin marks and gene expression profiles (Mahmoudi et al., 2019).

There are still only limited data from studies comparing the starting somatic cells to the matched iPSC-derived cell types and these have revealed both striking similarities, as well as some differences (Handel et al., 2016; Volpato and Webber, 2020). The latter may be related to the in vitro culture, the reprogramming and/or the developmental stage captured upon differentiation. Protocols of iPSC differentiation mimic development and coax the cells, through exposure to growth factors and morphogens, to exit pluripotency and commit gradually to a germ cell layer and a tissue-specific progenitor lineage. There is ample evidence that iPSC-derived cell types are characterized by a certain degree of developmental immaturity (Zeltner and Studer, 2015). Specifically, hematopoietic cells derived from iPSCs more closely resemble fetal than adult-type hematopoietic stem and progenitor cells (Ditadi et al., 2017; Lacaud and Kouskoff, 2017; Ivanovs et al., 2017). Notably, fetal liver hematopoietic stem/progenitor cells are characterized by remarkable proliferation and expansion properties. Thus it is possible, although it remains speculative, that the potent “stemness” of iLSCs is at least in part endowed or unleashed by the reprogramming and/or differentiation processes themselves by means that may involve “rejuvenation” or activation of fetal hematopoiesis programs. However, it should also be noted here, that reprogramming and re-differentiation of normal hematopoietic cells typically yields hematopoietic progenitors with more limited proliferation capacity than primary hematopoietic progenitor cells. Thus, the effects, if any, of the reprogramming and differentiation processes in increasing self-renewal of AML cells must be somehow specific to a leukemic context.

**Can iLSCs inform the generation of normal engraftable hPSC-derived hematopoiesis?—**The lack of engraftment of hPSC-derived hematopoiesis presents a major roadblock for both disease modeling and cell therapy applications of stem cell research. Despite intense research efforts over the years, the consistent and efficient generation or long-term repopulating HSCs with multilineage reconstitution potential from hPSCs is still elusive (Vo and De Daley, 2015; Papapetrou and Sadelain, 2010). While the reasons for this are not entirely clear, it seems to be a limitation of current differentiation protocols and not due to a lack of HSC potential by hPSCs, as HSCs with long-term repopulating capability can be generated in vivo from human iPSC-derived teratomas (Amabile et al., 2013; Suzuki et al., 2013). Despite our incomplete understanding of the nature of the problem, it is almost certain that a better understanding of the gene regulatory networks that sustain self-renewal of HSCs is needed to guide improvements in methods for producing HSCs from hPSCs. It is thus remarkable that AML-iPSC-derived hematopoietic cells can engraft so efficiently. This is currently the only case of robust, high-level, long-term engraftment of hematopoietic cells derived from any type of hPSC solely through directed differentiation without the forced expression of transcription factors or other exogenous factors (Kotini et al., 2017; Chao et al., 2017). iLSCs may thus provide a unique opportunity and a valuable tool to investigate the engraftment requirements of hPSC-derived cells and apply this knowledge towards the generation of engraftable HSPCs from normal hPSCs. It is possible, even though speculative at the moment, that the iLSCs may be

capturing a pre-HSC state that transiently emerges during hPSC directed differentiation and is rendered self-propagating and thus observable through an effect of the leukemic genome in enhancing the self-renewal of this cell state. While heavily myeloid-biased, these AML-iPSC lines could generate other lineages, such as erythroid, megakaryocytic (in vitro) and B lymphocytes (in vivo) (Kotini et al., 2017). Our study of iLSCs with integrated genomics approaches nominated several putative regulators of engraftable hematopoiesis that can be tested in future studies.

### 2.2. Opportunities for therapeutic targeting of AML LSCs

The therapeutic premise of the CSC model lies in the presumption that CSCs are the main source of cancer resistance and relapse. It is further assumed that CSCs are generally chemoresistant and that most current chemotherapies and targeted therapies do not target CSCs. CSCs are thought to have distinct biological properties than the bulk cells of a tumor that make them resistant to most conventional therapies, but some of which may create specific vulnerabilities that may offer opportunities for therapeutic targeting. Such therapies specifically targeting CSCs hold the promise of improved long-term outcomes or even cures. Better characterization of the distinct molecular circuitries that sustain CSCs could thus pinpoint new therapeutic targets specific to CSCs.

**Separating genetic from non-genetic sources of tumor heterogeneity.—** Heterogeneity that can be explained by the LSC model is strictly and necessarily non-genetic. The premise of the LSC model, as originally articulated in the 1990s, and of the CSC model more generally, is that cancers are hierarchically organized as a result of non-genetic determinants and that cells with LSC properties exist at a distinct epigenetic state within a genetically homogeneous cancer cell population (Dick, 2008). However, it is now apparent that genetic heterogeneity is an important source of heterogeneity, confounding the LSC model of non-genetic heterogeneity and the study of LSC properties (Kreso and Dick, 2014; Shackleton et al., 2009). Most LSC and CSC studies do not account for genetic heterogeneity, which makes claims on non-genetic sources of heterogeneity difficult to conclusively ascertain. By uniquely offering genetically clonal conditions, our iLSC model unequivocally lends support to the LSC model, as a source of non-genetic heterogeneity. Furthermore, it offers the opportunity to disentangle genetic from non-genetic contributions to phenotypic, epigenetic and functional heterogeneity. Different AML genetic clones are likely to differ in their LSC potential and frequency. PDX studies have revealed that different AML genotypes and subclones exhibit a high degree of variation in their engraftment potential (Klco et al., 2014). Furthermore, a recent seminal study by the Dick lab showed that the AML genotype can impact the LSC hierarchy, with some AML types exhibiting more “shallow” or “deep” hierarchies (Shlush et al., 2017). The future generation of AML-iPSCs from diverse AML genotypes capturing different genetic groups, clones and subclones, can help better characterize the contribution of genetics to the LSC hierarchy.

**LSC gene signatures.—**The clear associations of LSC frequency with clinical outcomes in AML patients and the impracticality of assessing LSC activity functionally in the clinical setting led several efforts to derive LSC gene expression signatures that can be used as surrogate measures of LSC activity (Eppert et al., 2011; Ng et al., 2016; Lechman et al.,

2016; Gentles et al., 2010; Jung et al., 2015). Gentles et al. derived a 52-gene LSC signature by sorting CD34+/CD38− cells from AML patient cells (without functional validation) and microarray gene expression analysis (Gentles et al., 2010). Another research group integrated DNA methylation with gene expression to derive a 71-gene LSC epigenetic signature using xenotransplantation-validated AML LSCs and identified the HOXA cluster as a key regulator of the LSC state (Jung et al., 2015). Ng et al. derived a 17-gene LSC gene signature (LSC17) from xenotransplantation-validated LSC-enriched populations using a statistical regression algorithm. The LSC17 score was prognostic of therapeutic resistance and overall survival in independent patient cohorts of diverse AML genetic groups (Ng et al., 2016). This LSC17 score or scores of subsets of the 17 genes were also shown to be predictive of prognosis in pediatric AML and MDS patient cohorts (Duployez et al., 2019; Wang et al., 2020). Among the 17 genes, GPR56, has also been independently identified as a AML LSC marker gene (Pabst et al., 2016). The same group has also generated a microRNA (miRNA) AML LSC signature from functionally validated LSC populations of AML patients and identified a role for a specific miRNA, miR-126, in LSC quiescence and self-renewal (Lechman et al., 2016). In our study, by integrating transcriptomic (RNA-Seq) and chromatin accessibility (ATAC-Seq) data, comparing the iLSCs to their genetically identical more mature progeny (iBlasts), we derived a 42-gene signature. This was further refined to a set of 16 genes that were non-overlapping with the LSC17 score and could predict survival in two independent AML patient cohorts (Wesely et al., 2020).

LSC signatures are limited by the degree of purity of LSC isolation. As stated earlier, no marker or combination thereof can isolate LSCs to a high degree of purity. Since the iLSCs in our study appear to be highly enriched for LSC activity, it is possible that the iLSC gene signature may capture a more faithful LSC program, but this still remains to be tested.

Upregulation of early hematopoiesis programs and Homeobox family genes were recurring themes observed in the various LSC signatures discussed above. Furthermore, some of these studies, including ours, showed that LSC gene signatures are predictive of disease outcomes across AML genetic groups, consistent with measures of LSC activity being an independent prognostic factor, although this requires further investigation. It still remains to be seen how useful such LSC signatures will be in the clinic, how they correlate to different genetic groups, especially those clearly associated with adverse or favorable prognosis, and whether they will provide additional prognostic information over currently used schemes to aid better classification and prognostication for AML patients. On the other hand, the various LSC gene sets, derived from transcriptomics, chromatin accessibility, miRNA or DNA methylation datasets, nominate new genes with a role in LSCs and can thus lead to new hypotheses and open new lines of investigation, as our study and other studies mentioned here, have highlighted (Pabst et al., 2016; Lechman et al., 2016; Wesely et al., 2020; Jung et al., 2015).

**New LSC targets.—**The importance of the LSC model in the clinic lies in the hypothesis that LSCs must be eradicated for long-lasting remission and that it should be theoretically possible to exploit specific vulnerabilities to uncover therapeutic targets. LSCs are believed to have properties distinct from those of the bulk AML cells that may provide opportunities to develop therapies that are targeted specifically against LSCs. Cellular properties, such as

drug efflux, DNA damage response and quiescence, may underlie chemoresistance of LSCs and at the same time provide new targetable vulnerabilities. LSCs have been shown to be quiescent and less susceptible to chemotherapy (Pollyea and Jordan, 2017; Ishikawa et al., 2007; Guzman et al., 2001; Guan et al., 2003; Hope et al., 2004; Terpstra et al., 1996; Saito et al., 2010). However, contradictory evidence also exists for the existence of cycling LSC populations with sensitivity to cytarabine similar to that of the bulk blasts, suggesting that these may not be universal properties of all AML LSCs (Eppert et al., 2011; Pollyea and Jordan, 2017; Sarry et al., 2011; Ho et al., 2016; Iwasaki et al., 2015). Metabolic and signaling dependencies have been proposed to potentially distinguish LSCs from the bulk of AML cells (Pollyea and Jordan, 2017; Thomas and Majeti, 2017b). These include oxidative phosphorylation, fatty acid oxidation, glutathione metabolism, mitochondrial activity, the unfolded protein response, heat shock protein response, WNT/β-catenin, NOTCH, Hedgehog and NF-kB pathway activation (Guzman et al., 2001, 2002, 2005; Lagadinou et al., 2013; Ye et al., 2016; Zong et al., 2015; Kagoya et al., 2014; Pei et al., 2013; Skrtic et al., 2011). Epigenetic dependencies of LSCs, such as DOT1L and LSD1 inhibition, have been proposed, especially for MLL-rearranged AML (Bernt et al., 2011; Harris et al., 2012; Schenk et al., 2012). Some therapies that are currently in development and proposed to target LSCs include Bcl-2 inhibitors, DOT1L and LSD1 inhibitors and BET inhibitors (Pollyea and Jordan, 2017; Lagadinou et al., 2013; Bernt et al., 2011; Harris et al., 2012; Schenk et al., 2012; Fong et al., 2015).

Cell surface markers that could distinguish LSCs from the bulk AML and from normal HSCs are highly desirable and currently an area of intense investigation. Such markers could be valuable as they could provide biomarkers to monitor response to therapy and predict relapse; targets for antibody-based (toxin-conjugated or eliciting cell-mediated toxicity) or CAR T-cell-based therapies; as well as a means of purifying LSCs for study. LSCs are generally enriched within the $CD34^{+}/CD38^{-}$ fraction, although it is now clear that other fractions contain LSCs as well (Bonnet and Dick, 1997; Eppert et al., 2011; Ishikawa et al., 2007; Sarry et al., 2011; Ho et al., 2016; Goardon et al., 2011). The interleukin-3 receptor α CD123 was the first marker proposed as a LSC specific antigen and a toxin-conjugated antibody against it is currently tested in clinical trials (Jordan et al., 2000). Other antigens currently tested in clinical trials as LSC specific targets are CD47 and CD33 (Taussig et al., 2005; Theocharides et al., 2012; Walter et al., 2012). CD99 and TIM3 are some more recent candidates (Jan et al., 2011; Chung et al., 2017). However none of these are exclusive or universal LSC markers and high variation in their expression has been observed between patients, within the same patient, as well as pre- and post- treatment (Pollyea and Jordan, 2017; Thomas and Majeti, 2017b).

Our iLSC study revealed dependence on the RUNX1 TF as a vulnerability of LSCs. By characterizing the relevant RUNX1 target genes, we discovered that TSPAN18 (Tetraspanin 18) at least partially mediates the RUNX1 effects in iLSCs. As a cell surface protein, Tetraspanin 18, can function both as a marker for purification, as well as a target for antibody or CAR-based approaches. Tetraspanins have diverse roles in membrane organization and compartmentalization, but also in cell signaling (Hemler, 2005). Other members of the tetraspanin family have previously been proposed to play roles in AML LSCs (Kwon et al., 2015; Vetrie et al., 2020; de Boer et al., 2018). The iLSC model could be

used to uncover and validate additional targetable genes, pathways or cellular processes specific to LSCs, although it still remains to be seen if any will sufficiently distinguish between LSCs and the bulk AML cells, as well as normal HSCs for a favorable toxicity profile.

**The role of RUNX1 as tumor suppressor and oncogene.—**Our analyses, integrating bulk RNA-Seq, single-cell RNA-Seq, ATAC-Seq and CHIP-Seq data, combined with RUNX1 knockdown (KD), zeroed in on RUNX1, as a critical TF for iLSCs. This was validated by functional experiments, showing that iLSCs, but not their differentiated progeny, the iBlasts, are dependent on RUNX1, since RUNX1 reduction by 50% abolished their leukemia initiating ability in vivo and led to their exhaustion in vitro (Wesely et al., 2020). By knocking down RUNX1 in primary AML samples we showed the generalizability of RUNX1 dependency across genetic subtypes of AML in vitro and in vivo. We did not find evidence for preferential RUNX1 dependency or lack thereof in association with any specific genetic group, but rather found that vulnerability to RUNX1 KD is determined by the LSC frequency (CD34+ fraction), consistent with the idea that RUNX1 is an LSC-specific dependency. We also derived a RUNX1-dependent LSC gene signature consisting of 20 target genes activated by RUNX1 (down after KD) and 47 genes repressed by RUNX1 (up after KD). This RUNX1-LSC signature correlated with patient survival and RUNX1 expression in AML patients (Wesely et al., 2020).

RUNX1 is a key transcriptional regulator of the hematopoietic system during development and the endothelial-to-hematopoietic transition (Gao et al., 2018; Chen et al., 2009). RUNX1, also known as acute myeloid leukemia 1 protein, AML1, has well-known tumor suppressor roles in AML. It is often translocated in sporadic AML and is the target of somatic and germline loss-of-function or dominant negative mutations, causing familial predisposition to AML (RUNX1-familial platelet disorder, FPD) (Song et al., 1999; Hatlen et al., 2012; Sood et al., 2017). On the other hand, a requirement for wild-type RUNX1 has been proposed in some types of AML, namely those harboring RUNX1 translocations, MLL translocations or FLT3-ITD mutations (Sood et al., 2017; Goyama et al., 2013) and an oncogenic role of RUNX1 in cooperation with FLT3-ITD has also been reported in a mouse model of AML (Behrens et al., 2017). Our results, implicating RUNX1 in LSC maintenance, can provide a mechanistic explanation for previous findings correlating high RUNX1 expression in AML patients with worse survival (Fu et al., 2016), a correlation that we also found in our analyses of independent AML patient cohorts (Wesely et al., 2020).

It is intriguing and somewhat paradoxical that RUNX1 haploinsufficiency or loss of function is an AML- initiating and promoting event and at the same time high RUNX1 levels are required for LSCs once AML is established, as haploinsufficiency for RUNX1 led to loss of LSCs over time (Wesely et al., 2020). There are several studies suggesting that RUNX1 dosage may be critical and that its levels need to be maintained within a certain range (Morita et al., 2017; Mill et al., 2019; Batcha et al., 2019; Antony-Debre et al., 2015). It is also possible that RUNX1 plays different roles at different stages during AML development, as it does during different stages in the development of the hematopoietic system. Very likely this TF holds more secrets that future studies will need to uncover. Before a better understanding of the role RUNX1 plays in different cellular contexts, ranging from normal,

pre-malignant and malignant hematopoietic cells can be attained, our findings suggest that approaches aimed at restoring RUNX1 expression in familial and sporadic cases of RUNX1-mutated AML should proceed with caution.

## 3. Concluding remarks

Even though the rationale for targeting LSCs to achieve long-lasting remissions or cures remains to be proven, targeting LSCs is a highly desirable goal of translational AML research. iPSC-derived LSC models offer new opportunities to understand the biological properties of LSCs, their dependencies on different AML genotypes and the exciting prospect of performing high-throughput genetic screens, such as CRISPR knockout screens, or small molecule screens of libraries of compounds.

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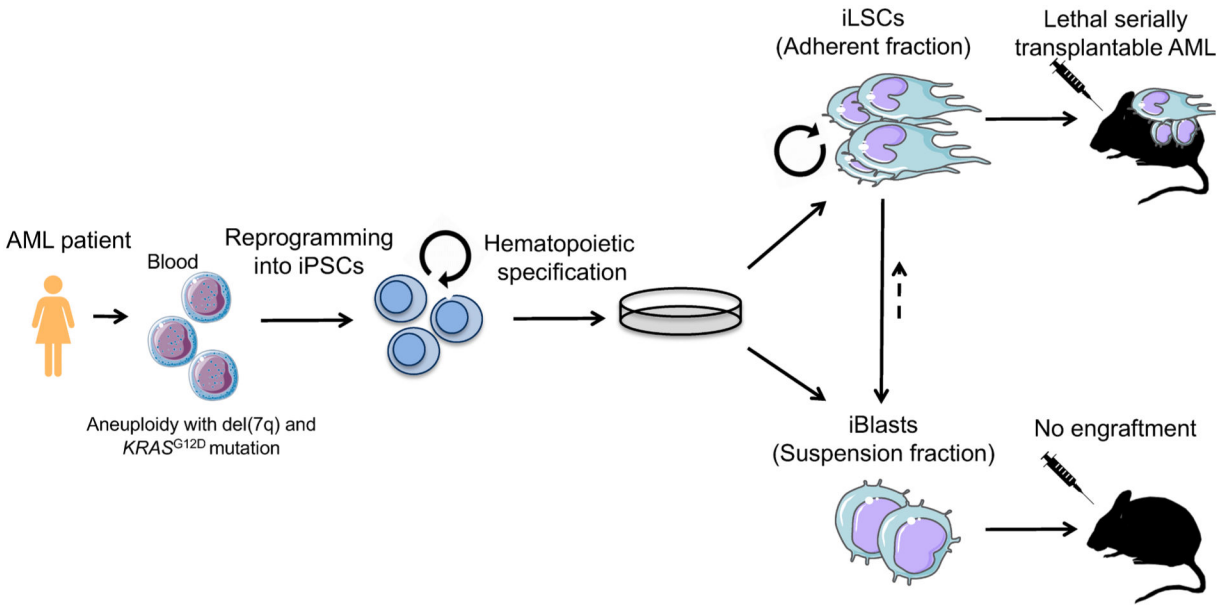


**Fig. 1. The iLSC model.**

Leukemic cells from a patient with AML with aneuploidy, del7q and a subclonal *KRAS*$^{G12D}$ mutation were used to generate iPSCs. Upon directed hematopoietic differentiation these exhibited phenotypic and functional heterogeneity consistent with an LSC hierarchy.