REVIEW

Understanding Human Oncogene Function and Cooperativity in Myeloid Malignancy Using iPSCs

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Myeloid malignancies are a spectrum of clonal disorders driven by genetic alterations that cooperatively confer aberrant self-renewal and differentiation of hematopoietic stem and progenitor cells (HSPCs). Induced pluripotent stem cells (iPSCs) can be differentiated into HSPCs and have been widely explored for modeling hematologic disorders and cell therapies. More recently, iPSC models have been applied to study the origins and pathophysiology of myeloid malignancies, motivated by the appreciation for the differences in human oncogene function and the need for genetically defined models that recapitulate leukemia development. In this review, we will provide a broad overview of the rationale, the challenges, practical aspects, history, and recent advances of iPSC models for modeling myeloid neoplasms. We will focus on the insights into the previously unknown aspects of human oncogene function and cooperativity gained through the use of these models. It is now safe to say that iPSC models are a mainstay of leukemia modeling "toolbox" alongside primary human cells from normal and patient sources.

HIGHLIGHTS

- Reprogramming of premalignant and malignant induced pluripotent stem cells (iPSCs) recapitulates leukemia development.
- iPSC models with diverse driver mutations provide insights into human oncogene function.
- Modeling sequential acquisition of mutations provides insights into oncogene cooperativity.

Induced pluripotent stem cells (iPSCs) are generated from somatic cells by reprogramming with the "Yamanaka" factors: OCT4, SOX2, KLF4, and MYC [1,2]. iPSCs can be differentiated into any somatic cell type and have been widely explored as a tool for disease modeling and cell therapies. Efforts to generate hematopoietic cells and hematopoietic stem cells (HSCs) from pluripotent stem cells began in earnest soon after the discovery of embryonic stem cells by Thompson et al. [3]. Wiles and Keller [4] reported that embryonic stem cells differentiated as embryoid bodies give rise to hematopoietic cells. Generation of bona fide HSCs from iPSCs has long been considered a major goal of regenerative medicine, and despite persisting challenges there is reason to believe that we are nearing this goal. On the disease modeling side, efforts quickly began to develop iPSC models of inherited Mendelian disorders, and more recently preleukemia and leukemia. Efforts to develop iPSC-derived models of hematologic disorders have been motivated by a number of key considerations. First, there has been a growing appreciation of the differences in human biology, oncogene function, and leukemia development [5,6]. Second, the recognition that normal and aberrant gene function needs to be defined in a proper stem or progenitor cellular context. Third, the need for genetically accurate models that capture the correct combination, allelic ratio, stoichiometry, and order of driver mutations. Finally, the need to generate large numbers of patient-derived cells for experimental purposes, such as genetic and drug screening. For a long time, the adoption of iPSCs has encountered roadblocks, including poor reprogramming efficiency, complex and expensive differentiation protocols, and failure to generate long-term transplantable HSCs. Despite these challenges, recent advances have motivated wider adoption of these models [7,8]. This includes the demonstration that most leukemias can be reprogrammed to pluripotency [9], acute myeloid leukemia (AML)-derived iPSCs recapitulate human AML *in vivo* [10–12], the widespread adoption of gene editing in iPSCs to generate fully isogenic models [10], the ability to reprogram genetically distinct subclones from individual patients [9,13], and numerous demonstrations that iPSC models inform previously unknown aspects of human pathophysiology that we aim to highlight in this review. It is safe to say now that iPSC models are a mainstay of leukemia modeling "toolbox" alongside primary human cells from normal and patient sources.

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0301-472X/

https://doi.org/10.1016/j.exphem.2024.104697

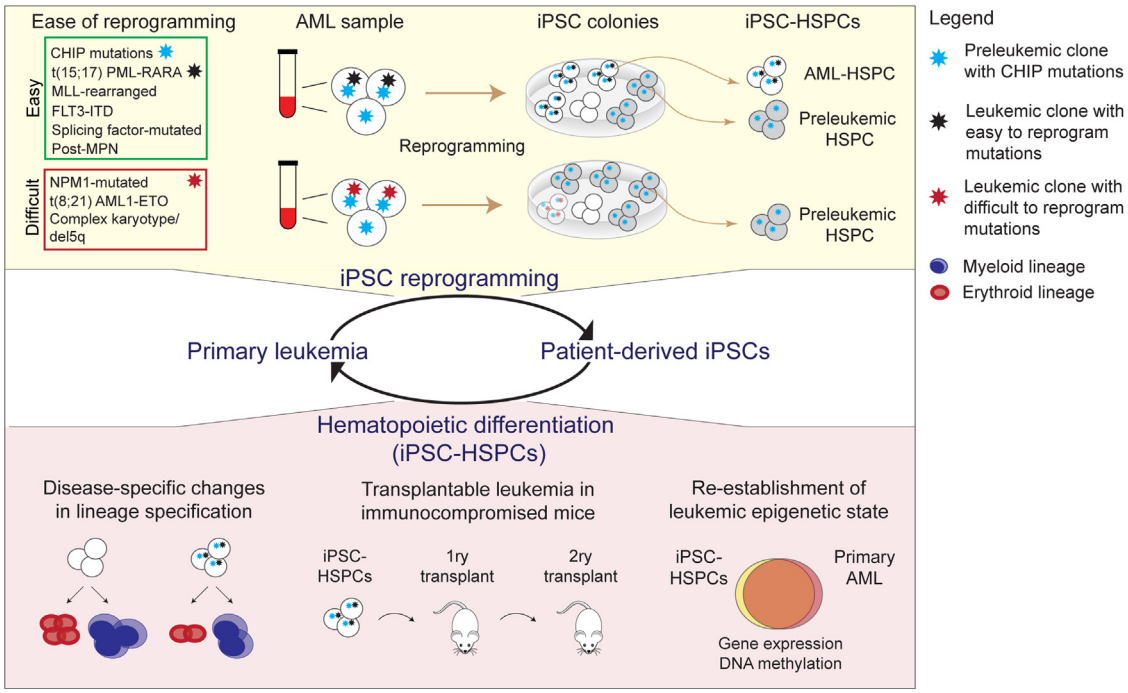

Figure 1 Induced pluripotent stem cells (iPSCs) reprogramming of primary leukemia samples and hematopoietic differentiation. The figure illustrates successes in establishing iPSC lines from acute myeloid leukemia (AML) samples of different genetic subtypes. Top: Genetic subtypes of AML are grouped into "easy-to-reprogram" (e.g., FLT3-ITD and PML-RARA) or "difficult-to-reprogram" (e.g., *NPM1*-mutated, complex karyotype). In addition to leukemic cells, samples of both subtypes may contain preleukemic clones with mutations commonly found in clonal hematopoiesis of indeterminate potential or CHIP (e.g., *DNMT3A, TET2*, and *TP53*). Easy-to-reprogram samples (top) efficiently give rise to iPSCs colonies and can be established into AML-HSPCs following hematopoietic differentiation. Difficult-to-reprogram samples (bottom) harbor leukemic mutations which decrease reprogramming efficiency and give rise to fewer iPSC colonies. However, preleukemic clones in the difficult-to-reprogram samples can still be reprogrammed because they lack the full set of leukemic mutations. Bottom: Following hematopoietic differentiation to iPSC-HSPCs, the hallmarks of successful re-establishment of leukemic state include changes in lineage specification, transcriptional and epigenetic similarity to original leukemia, and serial transplantation in immunodeficient mice.

BRIEF HISTORY OF LEUKEMIA REPROGRAMMING

A growing appreciation of the differences in the biology of human hematopoiesis and oncogene function have motivated attempts to reprogram human AML [7,8], summarized in Figure 1. The initial studies have reported reprogramming of specific genotypes of myelodysplastic syndromes (MDS) and AML. Chao et al. [11] and Kotini et al. [12] first demonstrated reprogramming of patients with MDS and AML with different genetic drivers. Importantly, hematopoietic stem and progenitor cells (HSPCs) derived from AML iPSCs could generate a serially engraftable leukemia in immunodeficient mice [11,12]. Because the reprogramming process results in the erasure of the somatic epigenetic state, these studies showed that the leukemia stem cell (LSC) state could be re-established upon hematopoietic differentiation of mutant iPSCs. However, in some cases, reprogramming efficiency was reported to be low, suggesting that only specific genotypes are amenable to reprogramming. There was a sense that reprogramming was an inefficient process that may be applicable to only specific genetic subtypes [14]. By reprogramming a large panel of human leukemias, Kotini et al. [9] recently demonstrated that most leukemic subtypes can in fact be reprogrammed to pluripotency. Moreover, following differentiation, iPSC-derived leukemic cells displayed remarkable molecular similarity to the leukemia of origin, which was further enhanced following engraftment in immunodeficient mice [9]. This study has demonstrated that iPSC models faithfully recapitulate the pathophysiology of human AML and the genetic complexity of human leukemias (Figure 1). It is likely that some "barriers" to reprogramming do exist, in cases when a somatic genotype is incompatible with reprogramming. This occurs for instance when specific driver lesion(s) impair gene function that is essential for the establishment or maintenance of pluripotent state. In such instances, when the leukemic genotype interferes with reprograming, iPSC colonies can be derived from preleukemic clones in the same patient sample. These clones carry fewer mutations and are often more likely to be reprogrammed. Hsu et al. [13] has shown that reprogramming routinely captures clonal intermediates that are present at low frequencies in the original patient sample. For example, aneuploid cells in MDS/AML with complex karyotype are rarely reprogrammed enabling the capture of preleukemic subclones with *TP53* mutations [13]. This allows us to establish the clonal order of mutations during disease progression and capture preleukemic clones as iPSCs for disease modeling (Figure 1).

DIFFERENTIATION OF NORMAL AND LEUKEMIC STEM CELLS

A dichotomy has emerged: iPSCs fail to generate normal HSCs during differentiation; by contrast, AML iPSCs can generate LSCs that re-establish leukemia upon transplantation. This shows that normal iPSC differentiation is dependent on growth factors and developmental cues in the culture media. By contrast, leukemic oncogenes can "instruct" LSC fate and epigenetic state largely irrespective of developmental cues. Given that preleukemic and MDS clones lack the full complement of leukemic driver mutations, these iPSCs also fail to generate transplantable cells. This is particularly important for modeling MDS, where few *in vivo* models exist. The development of differentiation protocols capable of providing more relevant cues remains a pivotal goal of the field. Initial efforts have focused on the use of transgenes to specify HSC fate. In the mouse, ectopic expression of HOXB4 was sufficient to specify HSC-like fate from iPSCs [15]. By contrast, *HOXB4* in humans is not essential for HSC patterning [16]. Instead, developmental specification of human HSCs requires distal *HOXA* activation including HOXA5, HOXA7, and HOXA9 [17,18]. HOXA activation is also a hallmark of human leukemias, mediated by MLL rearrangements and the NPM1c oncogene [19]. This demonstrates an important principle that factors required for HSC specification during development are tightly regulated in the adult, and their re-activation promotes aberrant HSC self-renewal and leukemic transformation. Leukemic oncogenes can enforce expression of HOXA, which explains why LSCs can be generated from iPSCs without exogenous factors. Doulatov et al. [20] showed that conditional expression of HOXA9, ERG, and other factors ("5F system") reactivates the HSC program during iPSC differentiation. This approach was later extended to generate transplantable HSCs by delivery of transgenes including HOXA5/9 to coincide with the onset of endothelial to hematopoietic transition [21].

Transgene delivery is difficult to make compatible with GMP-grade protocols for cell therapy. In recent years, the field has refocused on the development of "transgene-free" protocols. Although outside the scope of this review, important progress has been recently made toward this goal [22,23]. As an example, recent studies have uncovered an important role of retinoic acid in specifying HSC-competent hemogenic endothelium [24,25]. Incorporation of retinoic acid and other relevant developmental cues into differentiation protocols promises improved generation of transplantable HSCs [23,26]. Another recent advance is the development of iPSC-derived bone marrow organoids, three-dimensional (3D) structures which recapitulate the interactions between the malignant or premalignant clones and the marrow microenvironment [27].

TRANSGENE-BASED SYSTEMS AS EXPERIMENTAL MODELS

An important reason that has limited wider adoption of iPSC models is the technical difficulties in culturing and differentiating iPSCs. There is a plethora of hematopoietic differentiation protocols that utilize either embryoid body (or organoid) model or monolayer culture with or without supportive stroma. The comparison of current protocols has been reviewed elsewhere [28,29]. Most investigators recognize that protocols should incorporate mesoderm induction with BMP4 followed by induction of definitive hematopoiesis with Wnt agonists [30,31]. However, the timing and addition of specific morphogens and growth factors varies between protocols making it challenging for new investigators to adopt specific protocols. As discussed, recent advances promise to improve HSC specification with defined protocols. In the meantime, transgene-based systems such as the "5F system," that generate expandable iPSC-derived HSPCs can provide an important

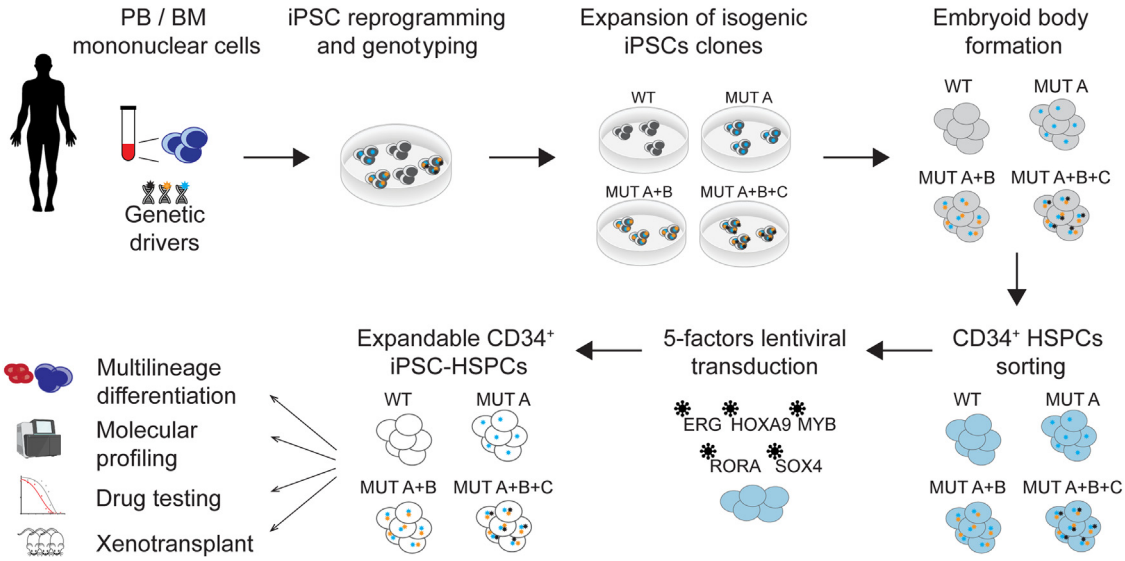

Figure 2 Schematic of the 5-factor system for generating expandable preleukemic induced pluripotent stem cells (iPSC)-derived hematopoietic stem and progenitor cells (HSPCs). In this system, multiple wild-type (WT) or mutant (MUT A, MUT A+B, etc.) subclones are first reprogrammed from patients with myeloid neoplasms and established into iPSC lines. iPSC lines are differentiated to CD34⁺ HSPCs following a defined hematopoietic differentiation protocol. Next, CD34⁺ HSPCs are transduced with lentiviruses conditionally expressing 5 transcription factors [20] and expanded for subsequent molecular and functional characterization, including multilineage differentiation, drug testing, and xenotransplantation.

resource for the community [7,20]. In this approach, iPSCs with specific combinations of driver mutations are reprogrammed, differentiated to $CD34^+$ HSPCs, and conditionally immortalized with five transcription factors (Figure 2). $CD34^+$ HSPCs are extensively expanded with doxycycline to drive transgenes using conventional cell culture methods, while retaining multilineage differentiation and *in vivo* transplantation potential upon doxycycline removal. This system provides a genetically accurate and tractable cell line model for studying specific driver mutations, individually and in combination with other drivers, especially when multiple preleukemic clones are available from an individual patient (Figure 2). Large numbers of cells generated using this system are compatible with *in vitro* and *in vivo* drug screening and testing. This transgene-based system may be a practical system for the wider hematology community.

SPECTRUM OF MYELOID MALIGNANCIES AND MUTATION COOPERATIVITY

Myeloid malignancies including myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS), and AML are clonal disorders marked by genetic alterations that drive aberrant self-renewal and differentiation [7,32–34]. Myeloid neoplasms are driven by recurrent germline and somatic mutations and chromosome abnormalities in >50 genes, including splicing factors, epigenetic regulators, signaling, transcription factors, and DNA damage response genes [35–38]. The origins of myeloid neoplasms are closely linked with clonal expansions or clonal hematopoiesis of indeterminate potential (CHIP), defined by the presence of a detectable clone (>2%) with one or more oncogenic mutations in the blood [39–42]. CHIP increases the risk of developing malignancy and is considered to be a common preleukemic state [43,44]. Initiating mutations found in CHIP often involve DNA methylation and chromatin modification genes (*DNMT3A, TET2*, and *ASXL1*), or DNA damage response (*TP53* and *PPM1D*) in specific (e.g., therapy-related) contexts [39–42]. Progression from CHIP to myeloid (or lymphoid) neoplasia is driven by acquisition of additional cooperating mutations, including genes involved in RNA splicing (*SF3B1, SRSF2*, and *U2AF1*), chromatin and genome organization (*IDH1*/2, *EZH2, STAG2*, and *NPM1c*), and others [45,46]. Late mutations that promote AML transformation, such as *FLT3, NRAS, KRAS,* or *PTPN11*, frequently involve activation of signal transduction pathways [37,47]. Chromosome abnormalities can be both initiating (e.g., 5q deletions) or late (17p deletions) depending on the context [48]. Recent single cell studies have refined these rules, for instance showing that *TET2* mutations can be both initiating and progression, and that late mutations often arise in multiple independent subclones [49,50]. It is now widely appreciated that the order and co-occurrence of driver mutations follows certain rules, however the principles of mutational cooperation have not been well defined. An important advantage of iPSC models is the ability to model mutation cooperation by direct reprogramming of pre-existing clones, CRISPR editing of *de novo* mutations, or a combination of both approaches in a genetically accurate, stoichiometric context. This is very important because many oncogenic mutations exert gain- or change-of-function effects and are highly dependent on proper gene dosage. Below, we will highlight some insights and principles of cooperativity gained from iPSC modeling of key oncogenic lesions in myeloid neoplasms, summarized in Figure 3.

SPLICEOSOME MUTATIONS AND ALLELIC STOICHIOMETRY

The discovery of driver mutations in the core spliceosome in human cancers is perhaps the most surprising outcome of sequencing cancer genomes [51]. The most frequent spliceosome mutations are in *SF3B1, SRSF2*, and *U2AF1* [35]. Seminal studies have shown that these are change-of-function mutations that alter gene splicing creating an array of mis-spliced gene products [52]. As a consequence, modeling these mutations is dependent on the correct stoichiometric ratio of mutant spliceosomes. A major unanswered question has been which gene targets cause different disease phenotypes, such as clonal dominance and ineffective erythropoiesis. iPSC models have been useful in untangling the complex genetics of splicing factor mutations. *SF3B1* mutations are found in ~30% of MDS and ~5% of AMLs, near-universally associated with the formation of ring sideroblasts, erythroid precursors with iron loading in a perinuclear mitochondrial ring [53,54]. Clough et al. [55] first developed *SF3B1*-mutant iPSC model (using the "5F system") that recapitulated the formation of ring sideroblasts during erythroid differentiation and showed that ring sideroblast formation is caused by mis-splicing of *ABCB7* and *TMEM14C* involved in heme and iron metabolism. iPSC models have uncovered new mis-splicing events induced by mutant SF3B1 and chromatin changes that promote erythroid-lineage bias in early progenitors [56]. Although *SF3B1* mutations are associated with favorable prognosis in MDS, *SRSF2*, and *U2AF1* portend poor prognosis. This led to a hypothesis that these mutations share common downstream target genes. Wheeler et al. [57] showed that *SRSF2* and *U2AF1* iPSCs promote alternative splicing of the long *GNAS* isoform resulting in increased ERK activation in mutant cells. Another recent hypothesis has proposed mechanisms unrelated to gene mis-splicing, including formation of DNA-RNA hybrid R-loops, detected in iPSC-derived models [58]. Current work centers on elucidation of the contributions of mis-splicing of target genes and non-splicing mechanisms to pathophysiology of these disorders.

GERMLINE *RUNX1* MUTATIONS AND GENE DOSAGE

Myeloid neoplasms with germline predisposition were first introduced in the 2016 World Health Organization (WHO) classification of myeloid neoplasms [59]. iPSCs are highly suitable for modeling germline disorders due to simplicity of reprogramming mutant clones from germline tissues (i.e., fibroblasts). In addition, iPSC differentiation traces the stages of embryonic hematopoiesis, capturing defects specific to fetal/neonatal hematopoiesis. Germline mutations in master transcription factor *RUNX1* cause familial platelet disorder (FPD), a classic inherited predisposition disorder. FPD has been extensively modeled using patient-derived iPSCs, uncovering a number of important findings. First, *RUNX1*-mutant, but not gene-corrected, iPSCs have defective platelet differentiation [60]. By contrast, overexpression of mutant *RUNX1* in wild-type (WT) iPSCs does not cause the same defect, and overexpression of WT *RUNX1* in mutant iPSCs restores most of the phenotype, demonstrating the loss-of-function type of these mutations [61]. Second, gene dosage of RUNX1 appears to be important. Antony-Debre et al. [62] showed that hemizygous dosage in iPSCs derived from patients with frameshift *RUNX1* mutations causes megakaryocyte defects, whereas lower (~20%) dosage or R174Q dominant-negative mutations promote

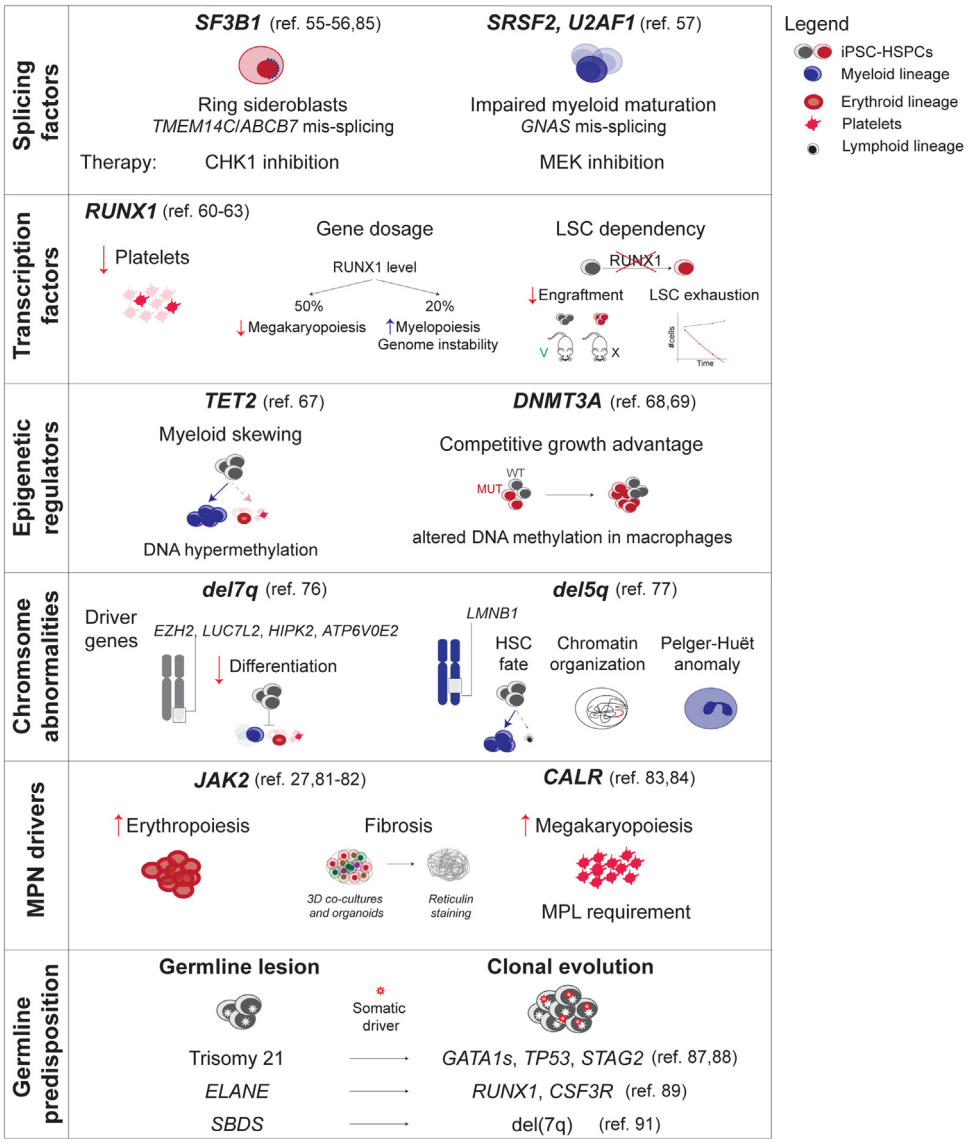

Figure 3 Characterization of the genetic drivers of myeloid neoplasms using induced pluripotent stem cell (iPSC) models. The figure illustrates the selected drivers of myeloid malignancies discussed in this review and insights into their molecular pathophysiology obtained using iPSC modeling approaches. These include oncogenic driver mutations in splicing (*SF3B1* and *SRSF2*) and transcription (*RUNX1*) factors, epigenetic regulators (*TET2* and *DNMT3A*), chromosome abnormalities and MPN drivers (*JAK2* and *CALR*). Additionally, iPSC models allow investigation of germline lesions predisposing to myeloid neoplasms (trisomy of chromosome 21, *ELANE,* and *SBDS* mutations) and somatic drivers of clonal evolution. For each mutation, the figure summarizes molecular or functional insights generated using iPSC modeling approaches in the referenced studies. A more complete description of the associated studies and their findings can be found in the corresponding section in the text.

myelopoiesis and genome instability. Although evidence generally suggests that *RUNX1* is a tumor suppressor, Wesely et al. [63] recently showed that the LSC program remains dependent on RUNX1. Taken together, modeling of genetically accurate mutations has highlighted that *RUNX1* may exemplify a class of tumor suppressors that do not follow the classic Knudson's two-hit model [64], but instead depend on precise gene dosage. Transformation may require reduced gene dosage but retains dependency on the functional gene product.

EPIGENETIC REGULATION BY *DNMT3A* AND *TET2*

Mutations in *DNMT3A* and *TET2* are the most frequent in CHIP and found in >50% of patients with MDS and AML. DNMT3A is a DNA methyltransferase that catalyzes the addition of a methyl group to cytosine residue of CpG dinucleotides, generally associated with gene silencing. TET2 converts 5-methylcytosine to 5-hydroxymethylcytosine (5hmC), generally promoting DNA demethylation and gene activation [65]. Despite seemingly opposing molecular functions, loss

of DNMT3A or TET2 both confer clonal fitness advantage to HSCs [65]. Intense efforts have been focused on understanding the molecular underpinnings of these functions. *DNMT3A* and *TET2* are also mutated in the germline setting, further supporting the use of iPSC models. Germline mutations in *DNMT3A* cause Tatton-Brown-Rahman syndrome (TBRS) an overgrowth syndrome [66], whereas germline *TET2* mutations cause immunodeficiency and predisposition to hematologic malignancies [67]. Stremenova Spegarova et al. [67] derived iPSC from patients with germline *TET2* mutations and showed that HSPCs displayed marked DNA hypermethylation and produced excess myeloid colonies. Modeling of *DNMT3A* mutations showed that HSPCs gain competitive advantage over normal HSPCs and partially recapitulate DNA methylation changes observed in AML [68]. iPSCs are a robust source of myeloid effector cells, such as macrophages and neutrophils. Interestingly, *DNMT3A*-mutant macrophages displayed both hypomethylation and hypermethylation at regulatory regions essential for immune function [69]. These findings suggest that DNMT3A loss may promote hypermethylation and silencing of active enhancers akin to TET2, although the mechanisms remain unclear. Epigenetic mutations are typically initiating lesions and rarely occur in isolation. The iPSC model lends itself well to cooperative modeling of epigenetic mutations with a defined mutation order.

CHROMOSOME ABNORMALITIES AND COOPERATIVE HEMIZYGOSITY

Chromosome abnormalities, including classic fusion oncogenes (*BCR/ABL1, AML1/ETO, PML/RARa*, etc.) and deletions of 5q, 7q, 17p, trisomy 8, and trisomy 21 are frequent in MDS and AML. By contrast with translocations which typically create two reciprocal fusion oncogenes, deletions or amplifications result in hemizygous gains or losses of a large number of genes. Deletion of large regions is likely favored in leukemic evolution because of concurrent hemizygous loss of multiple tumor suppressor genes which *cooperatively* promote transformation [70,71]. Identifying driver genes within recurrently deleted intervals is a classic problem in leukemia biology that remains largely unresolved. In some cases, the driver genes are apparent: for example, 17p loss encoding *TP53* is often found in cases with *TP53* mutations, fulfilling Knudson's two-hit model. In other cases, the drivers are much less apparent. Deletions of 5q (del5q) encode *RPS14, CSNK1A*, and *EGR1*, with known pathogenic roles [72–74]. Deletions of 7q encode *EZH2, CUX1*, and *LUC7L2* [75,76]. However, it is widely recognized that many unidentified driver genes are encoded in these regions. Given that chromosome structure is not well conserved across species, human cell models are needed. iPSCs represent an ideal model due to the availability of patient-derived lines with chromosome abnormalities and the ability to carry out functional genetic screens. Kotini et al. [76] first generated del7q iPSCs and showed that 7q loss blocked hematopoietic differentiation potential. Using functional genetic screens, the authors nominated *EZH2, LUC7L2, HIPK2*, and *ATP6V0E2* as 7q tumor suppressor genes. Reilly et al. [77] first utilized iPSCs with 5q deletion derived from a patient with *TP53*-mutant MDS and identified *LMNB1* encoding nuclear lamin B1 as the key 5q-encoded gene that regulates HSC function, genome stability, and Pelger-Huët nuclear anomaly (a common dysplastic phenotype in patients with MDS). These studies highlight the potential of iPSCs for discovery of new driver genes encoded in recurrently deleted chromosomal regions.

MPN AND NON-CELL-AUTONOMOUS EFFECTS

MPNs are clonal disorders driven by somatic mutations in *JAK2, CALR,* or *MPL*, activating cytokine signal transduction and promoting excessive cellular proliferation [78,79]. *JAK2* gain-of-function mutations, most commonly V617F variant, are present in >95% of polycythemia vera (PV) patients, and in about half of essential thrombocytosis (ET) and myelofibrosis (MF). Non-cell-autonomous phenotypes, critically marrow fibrosis, are hallmarks of MPN but have been challenging to model. Xenotransplantation of primary MF HSCs induces myelofibrosis *in vivo* [80]; however, primary patient samples are limiting; iPSC models can help fill this gap. Ye et al. [81] first developed an iPSC model of *JAK2* mutant MPN recapitulating enhanced erythropoiesis of primary PV. Flosdorf et al. [82] developed a 3D co-culture system with iPSC-derived megakaryocytes reproducing $JAK2^{V617F}$ induced fibrosis which was reverted by JAK inhibitor ruxolitinib. Khan et al. [27] recently developed a 3D organoid iPSC differentiation protocol which gives rise to hematopoietic, vascular endothelial, and stromal cell types. Seeding the organoids with MF patient-derived cells reproduced reticulin fibrosis in a TGFβ-dependent manner [27]. iPSCs reprogrammed from patients with *CALR* mutation showed enhanced megakaryopoiesis and identified the requirement for MPL in mutant CALR pathogenesis [83,84]. Together, these studies show that iPSC-based models can capture both cell intrinsic effects on the erythroid and megakaryocyte lineages, as well as non-cell-autonomous phenotypes including reticulin fibrosis, hallmarks of MPN pathogenesis.

IPSCS FOR DRUG DISCOVERY AND *IN VIVO* TESTING

An important application of iPSCs has been for compound and drug screening, owing to the genetically accurate context, availability of isogenic/gene-corrected controls, and large cell numbers for screening of small to medium-scale chemical libraries (on the order of thousands of compounds). A large number of reports have used candidate-based approaches for drug testing in iPSCs, leveraging the ability to derive genetically distinct clones. For example, Chao et al. [11] showed that *KRAS* WT clones are more resistant to cytarabine compared with *KRAS* mutant, consistent with *KRAS* dominance at diagnosis and absence at relapse in primary AML sample. Wang et al. [10] identified compounds targeting both early and late stages of leukemic evolution, including inhibitors of IRAK1/4 and UBE2N. *In vivo* testing of candidate compounds in the AML-LSC or the "5-factor" system can help overcome availability and heterogeneity of primary samples for proof-of-principle studies. Sarchi et al. [85] recently showed that *SF3B1*-mutant HSPCs generated using the 5-factor approach are selectively eradicated *in vivo* by treatment with CHK1 inhibitor prexasertib. Finally, medium-throughput chemical screening is an attractive use of iPSCs requiring large cell numbers. Chang et al. [86] screened ~2,000 compounds in MDS-derived iPSCs with del7q leading to the identification of niflumic acid, a cyclo-oxygenase-2 inhibitor, as selective inhibitor of del(7q) clones. We envision powerful applications of iPSC models in the near future for *in vivo* drug testing and rational or unbiased medium-throughput screening approaches.

MUTATION COOPERATIVITY AND ORDER IN LEUKEMIC TRANSFORMATION

The possibility of genetic manipulation and selection of genetically defined clones is a key advantage of the iPSC system. As such, it is well suited for modeling mutation order and cooperation. This can be done in two different approaches. The first approach is to take advantage of mutation order in the patients by direct reprogramming of clonal intermediates. Hsu et al. [13] importantly showed that iPSC reprogramming routinely captures premalignant clonal intermediates that remain in the marrow long after onset of frank neoplasm. From each patient, we can obtain a clonal series of isogenic clones with distinct combinations of mutations as well as isogenic WT iPSCs derived from normal cells in the patient sample. Hsu et al. [13] used this system to model the impact of sequential *SF3B1* and *EZH2* as well as *TP53* and del5q genetic lesions. An alternative approach is introduction of sequential mutations into iPSC lines from a normal individual by gene editing. Wang et al. [10] sequentially introduced an *ASXL1* truncation to model CHIP, *SRSF2* P95L mutation to model MDS, and *NRAS* G12D mutation to model AML transformation, culminating in transplantable AML. Often combining both approaches is useful, for example isolating premalignant clones by reprogramming and introducing additional mutations by gene editing. Kotini et al. [12] isolated premalignant clones with *GATA2* mutation and engineered del (7q) to model the mutation order in the patient. A combination of direct reprogramming with engineering of mutations provides a powerful system for establishing principles of mutation cooperativity, or conversely exclusivity, mutation order, and the cell of origin for malignant transformation.

Mutation cooperativity has been modeled in the context of germline predisposition, in which the initiating germline hit cooperates with the sequential somatic mutations. Individuals with Down syndrome (DS) and trisomy of chromosome 21 often develop myeloproliferative disorder driven by *GATA1* mutations (termed GATA1s), with additional somatic mutations promoting the development of acute megakaryoblastic leukemia (AMKL). Arkoun et al. [87] modeled this sequential process starting with trisomy 21 patient iPSCs and introducing GATA1s, *SMC3*, and *MPL* mutations, frequently found in DS AMKL. SMC3 loss cooperated with GATA1s to impair megakaryocyte maturation and dysregulate GATA1 target genes, which was enhanced in the trisomy 21 background. The addition of *MPL* mutation promoted growth factor independent growth [87]. Chen et al. [88] similarly started with reprogramming of trisomy 21 lines and introducing GATA1s, *TP53*, or *STAG2* mutations. They observed increased genome instability and accumulation of copy number variants linked to the amplification of *DYRK1A* in trisomy 21 HSPCs [88]. Secondary somatic *RUNX1* mutations are found in ~5% of AMLs and confer poor prognosis. Notably, secondary *RUNX1* mutations are found in nearly half of leukemias arising from congenital neutropenia (CN), a germline predisposition disorder caused by familial mutations in *ELANE* and other neutrophil genes. Dannenman et al. [89] established a model of stepwise CN-derived leukemogenesis by deriving *ELANE*-mutant iPSCs from CN patients and introducing secondary *CSF3R* and *RUNX1* mutations by CRISPR editing to uncover a critical dependency on the LSC gene *BAALC* during leukemic transformation. Shwachman-Diamond syndrome (SDS) is characterized by hematologic dysfunction and pancreatic insufficiency due to mutations in *SBDS* gene. SDS iPSCs have revealed elevated protease activity potentially linking multi-organ phenotypes in this disorder [90]. Ruiz-Gutierrez et al. [91] modeled MDS progression by engineering del(7q) into SDS iPSCs and showed that these disease stages display differential TGFβ activity which can be therapeutically targeted. Fanconi anemia (FA) is an inherited BM failure syndrome with extra-hematologic manifestations and predisposition to myeloid neoplasms. Marion et al. [92] investigated clonal evolution in an iPSC model of FA finding that *RUNX1* mutations mitigate quiescence to promote transformation of FA HSPCs. These studies highlight important insights into oncogene cooperativity garnered using the iPSC model system.

CONCLUSIONS AND PERSPECTIVES

As the hematopoietic differentiation protocols continue to improve, iPSC models will be an essential part of the leukemia modeling "toolbox" to understand the human oncogene function and cooperativity.

Conflicts of Interest Disclosure

The authors declare no potential conflicts of interest.

Acknowledgments

S.D. is supported by the NIH/NHLBI (R01 HL151651), NIH/NHLBI (R01 HL169156), NIH/NIDDK (RC2 DK127989), NIH New Innovator Award (DP2 HL147126), Wayne D. Kuni and Joan E. Kuni Foundation Discovery Grant, and Edward P. Evans Foundation Discovery Research Grant. S.D. is a Scholar of the Leukemia and Lymphoma Society (1391-24).

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