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Induced pluripotent stem cells and hematological malignancies: A powerful tool for disease modeling and drug development

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ABSTRACT

The derivation of human pluripotent stem cell (iPSC) lines by *in vitro* reprogramming of somatic cells revolutionized research: iPSCs have been used for disease modeling, drug screening and regenerative medicine for many disorders, especially when combined with cutting-edge genome editing technologies. In hematology, malignant transformation is often a multi-step process, that starts with either germline or acquired genetic alteration, followed by progressive acquisition of mutations combined with the selection of one or more pre-existing clones. iPSCs are an excellent model to study the cooperation between different genetic alterations and to test relevant therapeutic drugs. In this review, we will describe the use of iPSCs for pathophysiological studies and drug testing in inherited and acquired hematological malignancies.

1. Introduction

In the early 2000s, the search for factors able to establish a pluripotent transcriptional identity in a somatic differentiated cell led to the groundbreaking discovery of induced pluripotent stem cells (iPSCs), by Yamanaka and Takahashi (Takahashi and Yamanaka, 2006). Four transcription factors (TF) were described as necessary for reprogramming: OCT4, SOX2, KLF4 and c-MYC, also called OSKM. This combination generated iPSCs from a range of mammalian cells, including human cells (Hochedlinger and Jaenisch, 2015). Since this landmark discovery, other combinations of TFs and/or miRNAs have been described, but they all activate an endogenous transcriptional network that includes OCT4 and SOX2, similar to the one identified in embryonic stem cells (Boyer et al., 2005; Wang et al., 2006).

The most attractive properties of iPSCs are self-renewal and their capacity to differentiate into any cell type, features that make them appealing for regenerative medicine, disease modeling and drug testing purposes. Originally, the reprogramming factors were delivered by retroviral vectors, but to avoid increased risk of insertional mutagenesis, safer systems have replaced this method now, including non-integrative single-stand RNA virus and mRNA.

Besides insertional mutagenesis, genomic alterations, including copy number variations (CNVs), arise during cell culture and are selected by long-term culture (Hussein et al., 2011). Indeed, the continuous stress associated to cell culture affects genomic stability (Merkle et al., 2017), although it is not specific to pluripotent stem cell culture protocols. Recently, single-cell analysis techniques concluded that most of the mutations in newly derived iPSC clones are already present in the starting cell populations, with only few arising *de novo* (Kwon et al., 2017). It is therefore recommended to consider the tissue mosaicism variable, when selecting for the cell population to reprogram (Vijg, 2014). Another key parameter is the donor age, as there is a linear relationship between age and mutation burden, at least in the hematopoietic compartment (Lo Sardo et al., 2017).

The field of hematology has been interested in utilizing this technology since the beginning, particularly for its clinical translation potential (Kim and Daley, 2009). Today, iPSCs are successfully employed as a powerful, human-relevant model for pathophysiological studies. Simultaneously, industry researchers are increasingly employing pluripotent cells for therapeutics development.

2. The importance of hematopoietic differentiation protocols

The impact of iPSCs on hematology and disease modeling depends on the use of differentiation protocols that are reliable, reproducible and scalable. Theoretically, a pluripotent stem cell should give rise to every cell type specified by the three germ layers, including hematopoietic stem and progenitor cells (HSPCs). In line with this assumption,

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functional progenitors with long term-reconstitution potential are present in teratomas, generated via injection of pluripotent stem cells in immune-deficient mice (Amabile et al., 2013). Thus far, *in vitro* generation of HSPCs with long-term reconstitution properties has been reported only after forced expression of seven transcription factors (Sugimura et al., 2017). While both results are exciting, there is still an unmet need for simpler protocols. Several strategies have been proposed: some teams recapitulate *in vivo* transitions without using external genetic elements, a framework called ***directed differentiation*** (Ditadi et al., 2016; Ivanovs et al., 2017). Alternatively, ***direct conversion*** (Wahlster and Daley, 2016) utilizes a combination of genetic and epigenetic manipulations to derive HSPCs with an expanded set of differentiation and self-renewal properties.

2.1. Directed differentiation

Directed differentiation relies on lessons learned from developmental biology: during embryogenesis, hematopoietic cells arise from the mesoderm, in spatially and temporally distinct waves. In the yolk sac, the first wave gives rise to primitive erythrocytes (Ery-P), macrophages and megakaryocytes, while definitive hematopoiesis occurs later and gives rise to hematopoietic stem cells (HSCs), which emerge from the aorta-gonad-mesonephros (AGM) region in the dorsal aorta. HSCs will later colonize fetal liver and bone marrow. Hematopoietic stem and progenitor cells originate from specialized endothelial cells, the hemogenic endothelium (HE), through a process called endothelial-to-hematopoietic transition (EHT) (Lacaud and Kouskoff, 2017). *In vivo*, the activation of the BMP4, WNT/β-catenin, FGF and Nodal-activin pathways are crucial for specifying the migrating mesoderm (patterning) (Chadwick et al., 2003; Kennedy et al., 2007; Pick et al., 2007) and positional gradients ensured by cell–cell interactions are required to activate these pathways. Most directed differentiation protocols can be reduced to two stages: mesoderm induction and hematopoietic specification. Notably, while primitive hematopoiesis depends on the timely activation of Nodal-Activin and the inhibition of WNT/ β -catenin, definitive hematopoiesis requires the activation of WNT and the blockade of Nodal-Activin. A combination of markers (KDR and Glycophorin A) exists to distinguish primitive ($KDR^{+}GPA^{+}$) from definitive progenitors ($KDR^{+}GPA^{-}$) (Sturgeon et al., 2014).

Although mesoderm patterning can be reproduced *in vitro*, HSC specification remains elusive. *In vitro* it is not yet possible to discriminate the yolk sac-derived HE (leading only to progenitors) from the AGM-derived HE (generating HSC) (Yoshimoto et al., 2011; McGrath et al., 2015), complicated by the fact that they are specified very closely in time. Identifying differences between the two HEs *a posteriori* may help: it is possible that the AGM-derived HE is actually a mesodermal intermediate with hematopoietic potential only. This would explain why lineage tracing experiments did not reveal endothelial cells derived from AGM-bound progenitors (Swiers et al., 2013). Ditadi and colleagues (Ditadi et al., 2015) isolated three populations *in vitro*, committed, respectively, towards hematopoiesis ($CD34^{+}CD73^{-}CD184^{-}DLL4^{-}$ cells), arterial ($CD34^{+}CD73^{+}CD184^{+}$ cells) and venous endothelium ($CD34^{+}CD73^{+}CD184^{-}$ cells). The hematopoietic-committed population proved to be endothelial-like immediately after isolation, but then displayed exclusively an *in vitro* hematopoietic phenotype, with multilineage potential (T-cell and myeloid differentiation).

Initial experimental systems for hematopoietic differentiation from human pluripotent stem cells were based on co-culture with murine stromal cells in serum-containing media in the presence of instructive cytokines (Kaufman et al., 2001; Vodyanik et al., 2005). These protocols allowed the differentiation of several populations, but lacked a fine-tuned control of the pathways involved in cellular fate instruction. The current paradigm relies on serum-free, chemically defined media, where the amount of each instructive signal can be controlled. Nevertheless, better *in vitro* assays for evaluating differentiation potential are required, as *in vivo* reconstitution assays remain technically difficult. One surrogate is the globin expression pattern: erythrocytes from primitive hematopoiesis express mostly embryonic globin (ε-globin), while definitive progenitors express high levels of fetal and adult globin (γ and β -globin). Another *in vitro* assay is the capacity to produce T-cells, a cell type not present in primitive hematopoiesis, and more stringently, B-cells (Hadland et al., 2017).

2.2. Direct conversion

Instead of recapitulating *in vivo* processes, several labs attempted to generate hematopoietic cells, and possibly HSCs, by inducing fate conversion by expressing TFs or epigenetic regulators in combination with instructive cytokines (Pulecio et al., 2014; Batta et al., 2014; Pereira et al., 2016; Sandler et al., 2014; Lis et al., 2017; Riddell et al., 2014). This approach has gained support for its simplicity, as it is not constrained by a step-wise differentiation protocol. Although direct conversion is not strictly dependent on a pluripotent stem cell source (as it is not recapitulating the normal development), it could be sensitive to the epigenetic landscape of the cell of origin. For example, a committed myeloid precursor or an endothelial cell may be better candidates than a neuron, as they are developmentally closer to HSCs. The use of single-cell resolution techniques, refined cell culture methods, and efficient gene transfer systems, will be the three key parameters to improve this approach.

However, we should keep in mind that the forced expression of multipotency-associated TFs and epigenetic regulators could be deleterious for the activation of differentiation programs. Importantly, many of the proposed TFs are known oncogenes or play a role in leukemogenesis, so their aberrant expression could have consequences for HSC fitness. Therefore, these protocols are not entirely appropriate for pathophysiological studies, particularly for disorders involving the factors used for cellular conversion.

3. Induced pluripotent stem cells as a model for hematopoiesis

There are several advantages in using iPSCs as a model for hematological diseases. The first advantage is the clonal nature of the reprogramming process, which can be used to study clonal evolution and isolate the impact of specific mutations. This is particularly true for malignant hematopoiesis, since reprogramming isolates hematopoietic clones as biological individual entities (Saliba et al., 2015; Kotini et al., 2015). Therefore, iPSCs are a valuable resource for the study of clonal epigenetic profiles (Kotini et al., 2015), drug sensitivity (Chao et al., 2017) and disease progression (Kotini et al., 2017), without the need for complex genomic manipulation or transgenic animal models. However, clonal reprogramming can also be a limitation, as it increases experimental variability (Kilpinen et al., 2017). Compared to animal models or primary cells, iPSCs are affected by the limited availability of cell lines, which can magnify variation normally diluted by larger cell line collections. Even iPSCs derived from the same individual can have genetic differences, usually due to mosaicism in the tissue of origin (Kwon et al., 2017; Lo Sardo et al., 2017; Young et al., 2012). Long-term culture is also introducing variability (Merkle et al., 2017), and appropriate caution is required for high-quality experimental design. Guidelines for clinical grade iPSCs have been drafted (Sullivan et al., 2018), and should inspire similar efforts for research-grade cell lines. A common solution to reduce variability is using cell lines derived from different patients sharing the same genetic abnormality. In this way, it is possible to identify common features, and validate any correlations by introduction of an unmutated copy of the candidate gene. This experimental design confirms the specificity of the phenotype and links it unequivocally to the genotype. More recently, the widespread use of genome editing enabled another approach: instead of developing impractical large collections of iPSC lines, it is possible to introduce mutations of interest into a single, well characterized, genetic background, therefore comparing cells that differ only in one or a few defined genes. The development of

these isogenic models (Hendriks et al., 2016) is now the gold standard, with elegant systems recapitulating not only small insertions and deletions, but also larger chromosomal rearrangements (Park et al., 2014; Zuo et al., 2017). When these techniques are used, especially if performed in multiple rounds, careful verification of any off-target effect is recommended.

A further strength of iPSC modeling is related to pluripotency: it is possible to differentiate them into different cell types and combine them in an artificial human hematopoietic niche. This system would be suitable for studying cellular interactions in a highly controlled setting and overcoming the limitations of co-culture systems based on murine cell lines. Conversely, the *in vitro* nature of iPSC technology prevents more comprehensive pathophysiological studies. This issue, common to other approaches (primary cell culture, immortalized cell lines and organoids), has been partially addressed by the generation of humanized niches *in vivo* in mouse models, both by tissue (Reinisch et al., 2016) and genome engineering (Rongvaux et al., 2014; Yurino et al., 2016). The latter approach, in particular, helped the study of specimens historically refractory to long-term engraftment (Yoshimi et al., 2017; Zhang et al., 2017).

Finally, the iPSC technology allows the generation of large numbers of more committed progenitors and precursors that faithfully recapitulate the *in vivo* counterparts, with a higher degree of similarity than any other human-relevant model. Since technical protocols for the derivation, maintenance and differentiation of iPSCs are now widely accessible, we can expect a steadily increasing number of reports using iPSCs to investigate the molecular mechanisms underlying hematological disorders.

4. Application of iPSCs in malignant hematological disorders

Malignant transformation starts with an inherited or acquired genetic alteration, followed by clonal selection and progressive increase of the mutational burden (Greaves, 2016). Hematological malignancies are classified by the World Health Organization (WHO) according to their clinical, morphological and molecular features, and major categories include myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) (Arber et al., 2016). Furthermore, increasing numbers of familial myeloid neoplasms are reported, with germline mutations affecting leukemia-predisposing genes. These cases typically emerge from pre-existing hematological conditions, like platelet disorder or neutropenia, or syndromes affecting multiple organs, like inherited bone marrow failure (BMF) syndromes. Disease modeling strategies using iPSCs derived from patients or healthy individuals combined with genome editing technologies are presented in Fig. 1 and described in the subsequent paragraphs.

4.1. Inherited predisposition to malignant hemopathies

One of the main advantages of iPSCs for this disease class is the generation of cell lines harboring the germline mutation alone or with additional mutations. Their comparison allows to study cooperation and how the germline hit influences disease evolution. Moreover, iPSCs are ideal to study the role of mutations in embryonic development and lineage specification. In the last decade, tens of iPSC lines have been established from patients harboring germline abnormalities affecting one or more hematopoietic lineages and associated to predisposition to

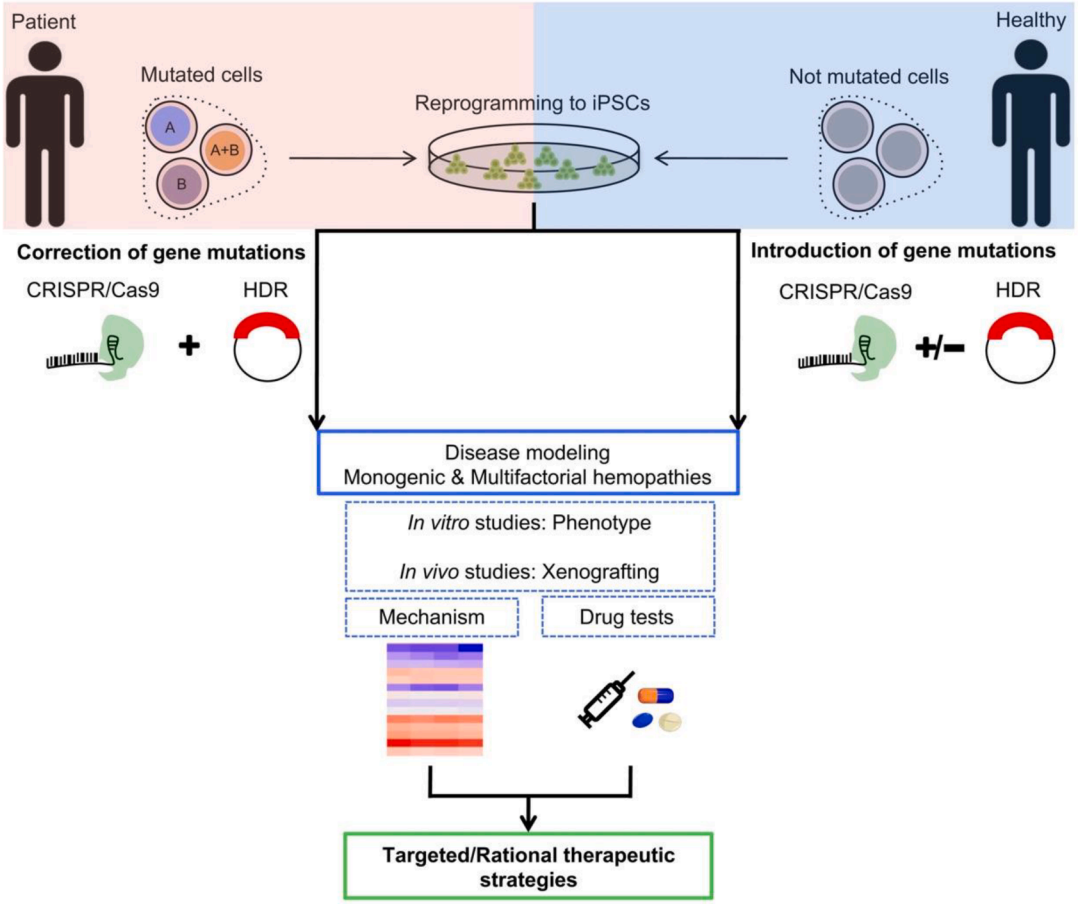

Fig. 1. Disease modeling strategies using iPSCs derived from patients or healthy individuals combined with genome editing technologies. A: Cell carrying mutation A; B: Cell carrying mutation B, A + B: Cell carrying both A and B mutations; CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats; HDR: Homology Directed Repair; iPSCs: induced Pluripotent Stem Cells.

MPN, MDS and leukemia. We will discuss the role of germline abnormalities linked to deregulated megakaryopoiesis, such as *RUNX1* mutation in FPD/AML, or inducing abnormal granulopoiesis, such as *GATA2* and *ELANE* mutations. Finally, we will discuss the modeling of BMFs that predispose to MDS/AML. Our goal is to highlight case studies and how iPSC modeling was central in these reports.

4.2. FPD/AML due to RUNX1 mutations

Familial platelet disorder associated to acute myeloid leukemia (FPD/AML) was one of the first platelet disorders modeled with iPSCs. It is an autosomal dominant condition due to mutations in the gene encoding the transcription factor RUNX1. Patients exhibit moderate thrombocytopenia, defects in platelet function and leukemia predisposition. Four studies showed that patient iPSCs recapitulate the defective megakaryopoiesis (Antony-Debré et al., 2015; Connelly et al., 2014; Iizuka et al., 2015; Sakurai et al., 2014), explaining the complete penetrance of the thrombocytopenia in FPD/AML. Antony-Debré *et al.* (Antony-Debré et al., 2015) demonstrated that the type of mutation affects the penetrance of leukemic predisposition. While the haploinsufficiency mutation *RUNX1*^{R139X} causes only defective megakaryopoiesis, the dominant negative (DN) *RUNX1*^{R174Q} mutation, found in families with higher number of leukemia cases, increases HSPC clonogenic potential and causes p53-dependent genomic instability. Both defects were corrected after re-introduction of *RUNX1*^{wt}. Moreover, the reduction in RUNX1 expression led to a highly comparable phenotype, suggesting that the DN *RUNX1*^{R174Q} mutation acts as a loss-of-function, and supports the model of RUNX1 dose-dependency as the main mechanism behind leukemic transformation.

4.3. Trisomy 21

Children bearing trisomy of chromosome 21 (also called Down Syndrome (DS)) present an increased risk (500-fold in the first two years of life) of developing acute megakaryoblastic leukemia (AMKL) (Hitzler and Zipursky, 2005). In addition, up to 10% of children display transient myeloproliferative disease (TMD). Both conditions are associated with somatic mutations in GATA1, leading to a truncated protein called GATA1short (GATA1s) (Rainis et al., 2003; Wechsler et al., 2002). Several studies successfully generated stable iPSCs from patients (Byrska-Bishop et al., 2015; MacLean et al., 2012; Park et al., 2008), and showed that GATA1s decreases erythropoiesis, to the benefit of myelopoiesis and megakaryopoiesis. They also demonstrated that the binding of GATA1s to erythropoietic target genes is specifically decreased and that the N-terminus of GATA1 is key for chromatin binding (Byrska-Bishop et al., 2015). Moreover, iPSCs have been used to study the role of trisomy 21 alone in embryonic and fetal hematopoiesis: in the former, it decreases erythropoiesis, increases myelopoiesis and has no impact on megakaryopoiesis (Chou et al., 2012), while in the latter, it increases the number of hematopoietic progenitors of all three lineages (MacLean et al., 2012).

4.4. Familial MPN with 14qCNV (ATG2B/GSKIP)

Most leukemic predisposition syndromes are characterized by moderate penetrance. However, the germline copy number variation (CNV) in 14q32 is a nearly completely penetrant MPN/leukemic predisposition syndrome. It consists of 6 duplicated genes, three of which (*TCL1A, ATG2B* and *GSKIP*) are expressed in hematopoietic cells (Saliba et al., 2015). In addition to the duplication, patients harbor somatic mutations in receptor-mediated signaling genes (*JAK2*^{V617F}, *CALR* or *MPL*) and epigenetic regulators (*TET2 or IDH1/2*). iPSCs from two patients from the same pedigree were reprogrammed, obtaining clones bearing only the CNV, the CNV and JAK2^{V617F} and the CNV with *JAK2*^{V617F} and *TET2*^{mut}. This allowed to model the disease progression and study the role of the CNV alone and in cooperation with mutations. The 14qCNV was shown to be sufficient to increase the production of hematopoietic progenitors and the production of erythroblasts, megakaryocytes and monocytes, an effect enhanced by *TET2*^{mut} and/or *JAK2*$^{WT/V617F}$ mutation. Moreover, the CNV promoted megakaryopoiesis and cooperated with *JAK2*^{V617F} mutation to modify the hematopoietic progenitor response to EPO.

4.5. Severe congenital neutropenia (SCN) due to ELANE mutation

Disorders of the granulocytic lineage are intimately linked to leukemic predisposition. The most well-known example is the case of severe congenital neutropenias (SCNs), a heterogeneous group of disorders characterized by impaired neutrophil maturation, with a block at the promyelocyte-myelocyte stage. Mutations in more than 25 genes have been described: the most frequent occur in the gene *ELANE* (40%) (Dale et al., 2000), encoding for neutrophil elastase (NE). Its role in SCN remains unknown, but seems to involve ER stress induction, which triggers the unfolded protein response (UPR) and leads to the maturation block. The cytokine G-CSF is currently used to treat neutropenia: while efficient in restoring normal neutrophil counts, long-term treatment increases the risk of malignant transformation (Rosenberg et al., 2006). A recurrent pattern has been described: mutations in the G-CSF receptor (*CSF3R*) arise early, followed by *RUNX1* mutations that effectively trigger the leukemic transformation (Touw, 2015). Mouse models mutated for *Elane* do not display a phenotype comparable to SCN, thus iPSCs were considered a suitable model. Cell lines derived from *ELANE*^{mut} patients (Hiramoto et al., 2013; Nayak et al., 2015) efficiently recapitulated the maturation defect. Hiramoto *et al.* (Hiramoto et al., 2013) reported reduced expression of genes of the Wnt3a/ β -catenin pathway and they demonstrated that *in vitro* Wnt3a administration improves neutrophil maturation via up-regulation of lymphoid enhancer-binding factor 1 (LEF-1). Nayak and colleagues (Nayak et al., 2015) showed that *ELANE* mutations caused NE mislocalization and UPR/ER stress. The use of a NE-specific small-molecule inhibitor (Sivelestat) corrected the defective maturation, restoring NE localization in primary granules and decreasing UPR/ER stress. Inhibitors of NE, acting either directly or as chaperones for the mutant protein, were also tested on iPSC-derived neutrophils: the inhibitor MK0339 was shown to improve cell survival and increase maturation of neutrophils (Makaryan et al., 2017). Finally, Dannenmann *et al.* (Dannenmann et al., 2019) derived clones with *ELANE* mutation only and with additional mutations (*CSF3R*; *RUNX1* and trisomy 21). In all conditions, they confirmed an elevated ER stress and UPR, increased susceptibility to DNA damage and delayed DNA repair. The same group also showed that *ELANE* knock-out in patient-derived iPSCs and primary cells restores neutrophil maturation (Nasri et al., 2019).

4.6. Congenital neutropenia due to GATA2 mutations

Heterozygous point mutations, small insertions or deletions in the *GATA2* gene cause an inherited disorder characterized by bone marrow failure, increased infection risk and predisposition to myeloid malignancies. GATA2 is involved in the generation and maintenance of HSCs, by promoting EHT in the AGM. Hematopoietic progenitor cells ($CD34^+CD45^+$ cells) differentiated from patient-specific iPSC lines harboring heterozygous mutations in the zinc finger domain or in the intron 5, did not reproduce patient abnormalities, and only subtle differences were observed compared to healthy controls. Conversely, using isogenic iPSCs with *GATA2* deletion revealed a decrease in $CD34^+CD45^+$ progenitor numbers in a dose-dependent manner, with almost no hematopoiesis in the total absence of GATA2. This suggested that one *GATA2* wild-type copy is sufficient, in patient iPSCs, to induce normal hematopoiesis (Jung et al., 2018). This study raised questions about the need for differentiation protocols able to produce adult HSCs and the necessity for secondary hits for the development of full-blown disease. For the latter question, iPSCs are unique tools, as cell lines

derived from patients undergoing malignant transformation are highly informative. This approach has been successfully exploited in a different study: cell lines were derived from a high-risk MDS patient with a germline heterozygous *GATA2* point mutation, an acquired small deletion on the second *GATA2* allele, a del(7q), and mutations in *U2AF1* and *ETV6*. The presence of all these alterations leads to impaired hematopoiesis, but no long-term engraftment in immunodeficient mice (Kotini et al., 2017).

5. Inherited bone marrow failures (BMFs)

Among all BMFs modelled with iPSCs, the most well-known is Fanconi Anemia (FA), caused by mutations in one of the sixteen *FANC* genes of the FA pathway, involved in DNA repair. Mutations in those genes lead to HSPCs exhaustion (Muller et al., 2012; Raya et al., 2009). While initial attempts to derive iPSCs from FA patients failed, some clones have been generated, albeit with reduced efficiency (Muller et al., 2012; Yung et al., 2013) and capacity to be maintained in culture (Yung et al., 2013), and a significant number of chromosomal aberrations (Liu et al., 2014; Yung et al., 2013). Delivering copies of the *FANC* gene either via integrative (Muller et al., 2012; Raya et al., 2009), non-integrative (Liu et al., 2014) or inducible (Bharathan et al., 2017) vectors markedly increased the reprogramming efficiency (Bharathan et al., 2017; Muller et al., 2012; Osborn et al., 2016). These complemented iPSCs were genetically stable and able to differentiate into multilineage HSPCs.

Another BMF that could evolve to MDS/AML is Shwachman-Diamond syndrome (SDS), caused by mutations in the *SBDS* gene (90%). This gene is localized on the long arm of chromosome 7 (7q11) and encodes a protein involved in ribosomal maturation (Boocock et al., 2003). The first SDS-iPSCs reproduced the disease and revealed a link between the hematopoietic defect and the exocrine insufficiency that leads to pancreatic dysfunction in SDS patients (Tulpule et al., 2013). It is worth noting that typical MDS/AML progression is often associated with clonal anomalies of chromosome 7 (Maserati et al., 2009), and chr7 monosomy or deletion of 7q are known to arise in high-risk MDS/AML. For these reasons, SDS-iPSC lines were developed with and without 7q deletion (Ruiz-Gutierrez et al., 2019). The iPSCs were derived from 2 patients with homozygous *SBDS* mutation and were further engineered to introduce the del(7q), proving the efficiency of combining iPSC reprogramming and genome editing for disease modeling.

6. Acquired malignant hemopathies

Although the first studies were mostly focused on monogenic disorders, iPSC technology is now applied to study more complex diseases, like acquired malignant hemopathies. In this context, the iPSCs clonal origin is advantageous, as reprogramming of a single sample could provide a snapshot of the patient clonal architecture. The iPSC lines could therefore be exploited to study mutation cooperation or therapy sensitivities.

6.1. Myeloproliferative neoplasms (MPN)

MPN are clonal malignancies that cause myeloid cell overproduction. MPN include chronic myeloid leukemia (CML), classical MPN, rare and unclassified MPN, chronic eosinophilic leukemia (CEL) and chronic neutrophilic leukemia (CNL).

6.2. Classical MPN

Classical MPNs (non BCR-ABL^{+}) include essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF), corresponding to the overproduction of platelets (ET), red cells (PV), granulocytes and megakaryocytes (PMF), respectively. Classical MPNs are due to acquired mutations resulting in constitutive activation of cytokine receptors/JAK2 and downstream signaling pathways. Three types of acquired mutations have been identified: *JAK2*^{V617F}, mutations in the thrombopoietin receptor *MPL* and in the calreticulin (*CALR*) genes. Heterozygous *JAK2*^{V617F} clones are found in ET cases, while homozygous *JAK2*^{V617F} clones mostly occur in PV, which underlies a link between JAK2 signaling intensity and disease phenotype. More than 50 *CALR* mutations have been described for ET and PMF, the most frequent being *CALR*del52 and ins5 (Vainchenker and Kralovics, 2017).

iPSCs generated from a PV patient (*JAK2*$^{wt/V617F}$) showed increased erythropoiesis and a gene expression profile comparable to the hematopoietic progenitors from the same patient (Ye et al., 2009). To understand the impact of genotype (heterozygous vs homozygous) on pathology, two groups have generated two different models: isogenic *JAK2*^{V617F} iPSCs (Ye et al., 2014) and *JAK2*^{V617F} iPSCs from different patients (Saliba et al., 2013). Both teams reported increased megakaryocyte, erythroblast and monocyte numbers for homozygous *JAK2*^{V617F}, faithfully recapitulating the disease. In addition, homozygous *JAK2*^{V617F} induced the growth of EPO- and TPO-independent erythroid and megakaryocytic progenitors, whereas *JAK2*$^{wt/V617F}$ induced spontaneous growth only of megakaryocytes. This explains why heterozygous *JAK2*^{V617F} is mainly associated with ET and homozygous *JAK2*^{V617F} with PV. Cells from PMF patients carrying *JAK2*^{V617F} and additional genetic abnormalities were also reprogrammed, recapitulating both megakaryocyte hyperplasia and higher IL-8 expression (Hosoi et al., 2014). Finally, iPSCs derived from a patient carrying *CALRins5* mutation displayed a TPO-independent growth of megakaryocyte progenitors in the presence of MPL, an increased number of megakaryocytes compared to controls, and a higher expression of GATA1 and GATA2, suggesting an increased megakaryocytic commitment of progenitor cells (Araki et al., 2016; Takei et al., 2018).

6.3. Chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML)

CMML is a disorder at the boundary between MPN and MDS: characterized by persistent monocytosis and defective monocytic differentiation, this hemopathy typically affects the most senior patients. Due to mutations in epigenetic/splicing/signaling/chromatin modifiers in an aging context, this disorder has always escaped a clear classification and represents an interesting case for the understanding of myeloid malignancies. The disease pathophysiology remains poorly understood, partly because of the lack of relevant animal models. Taoka and colleagues successfully derived multiple cell lines from a single patient (Taoka et al., 2018). HSPCs from those cell lines displayed proliferative monocytosis and serial replating, suggesting leukemic potential. In molecular terms, the authors detected constitutive activation of ERK, which was reversed with different inhibitors. Moreover, they established a humanized CMML mouse model via teratoma formation *in vivo*, as a preclinical model for drug testing (Taoka et al., 2018). Another team generated CMML-iPSCs from a patient carrying 12 mutations, including *KDM6A, TET2* and *KRAS*^{G12D} (Beke et al., 2020). Interestingly, some cell lines that displayed high similarity in genetic terms were markedly different in their differentiation potential. The authors linked this feature to a layer of heterogeneity independent from genetic cues, and probably associated to epigenetic differences.

While similar to CMML, JMML is exclusively associated with somatic and/or germline mutations in *CBL, NRAS, KRAS, PTPN11* or *NF1* genes, all involved in cytokine receptor signaling. iPSCs were derived from cells harboring somatic *PTPN11* (Gandre-Babbe et al., 2013) and *CBL* mutations (Gagne et al., 2018). These models efficiently recapitulated the pathological features of JMML, including increased myelopoiesis and constitutive GM-CSF activation. While PI3K and mTOR inhibitors inhibited signaling and myeloproliferation in the presence of both mutations, *PTPN11*^{mut} cells were more sensitive to MEK inhibitors, and *CBL*^{mut} cells to JAK inhibition (Tasian et al., 2019). Finally, iPSCs harboring germline *PTPN11* mutation displayed increased STAT5-mediated signaling, and upregulation of miR-223 and miR-15a

(Mulero-Navarro et al., 2015). In conclusion, all these models proved to be reliable for *in vitro* drug testing, particularly relevant for rare disorders like JMML, where there is a dearth of models for therapeutics development.

6.4. Myelodysplastic syndromes (MDS)

MDS are characterized by defective maturation of one or multiple myeloid lineages, which leads to anemia, thrombocytopenia and neutropenia. Kotini et al (Kotini et al., 2015) generated iPSC clones from two patients harboring del(7q), the most frequent chromosomal alteration. Those lines were deeply impaired in their hematopoietic differentiation, displaying reduced number of $CD34^{+}CD45^{+}$ progenitors. They were compared with healthy lines edited to carry deletions spanning different regions of the chromosome 7, in order to identify the region responsible for the MDS phenotype. They narrowed it down to the 7q32.3-7q36.1 as the culprit, and validated this hypothesis identifying four genes (*EZH2, LUC7L2, HIPK2* and *ATP6V0E2*) that were able to rescue the defective hematopoiesis. Of note, *EZH2* and *LUC7L2* loss-of-function mutations are also found in MDS and AML patients (Hosono, 2019). More recently, the same team reported the combination of del(7q) and $SRSF2^{P95L}$, an anomaly frequently found in MDS and CHIP (clonal hematopoiesis of indeterminate potential). They concluded that *SRSF2* mutation alone, contrary to del(7q) alone, contributes only slightly to the hematopoietic defect (Chang et al., 2018).

iPSCs has been used to model all the different steps of leukemogenesis, by the comparison of cell lines derived from progressively more advanced myeloid malignancies (Kotini et al., 2017). Although the lines were derived from patients with different genetic backgrounds, the authors made use of genome engineering to introduce some pathogenic alterations (del7(q)) in a specific genomic context. All the lines displayed progressively more impaired hematopoietic differentiation, an observation supported by the transcriptomic analysis. In conclusion, these cell lines represented specific steps of the myeloid malignant transformation, proving that a collection of iPSC lines could be exploited for screening molecules able to target specific stages of the myeloid leukemia transformation. This study also revealed that patient cells harboring del(5q)-MDS or monosomy 7 were impossible to reprogram, and the reprogramming was strongly impaired for del(7q), when compared to the original sample clonal architecture (Kotini et al., 2017).

7. Leukemias

7.1. AMl

While MDS reprogramming was limited in clonal representation, reprogramming AML blasts proved even more complicated, as these cells carry a larger mutational burden. Various teams tried to derive AML-iPSCs (Chao et al., 2017; Hoffmann et al., 2016; Kotini et al., 2017; Lee et al., 2017). Bhatia and colleagues tried to reprogram over thirteen genetic subtypes of AML, but were successful only in one patient harboring the MLL-AF9 rearrangement (Lee et al., 2017). Contrary to normal, preleukemic, “low-risk and “high-risk” MDS, HSPCs differentiated from AML-iPSCs were able to engraft a serially transplantable leukemia in immunodeficient NSG mice, and proved to be an efficient preclinical model for drug testing. Moreover, when in a pluripotent state, reprogrammed AML-iPSCs lost the leukemia-specific epigenetic abnormalities, resetting their epigenetic status. However, hematopoietic differentiation allowed the re-acquisition of the leukemic epigenetic landscape, confirming that the malignant program is driven by the overarching hematopoietic transcriptional programs.

7.2. Pediatric leukemias

Pluripotent stem cells may be very effective models for pediatric leukemias, as they reproduce the earlier waves of hematopoiesis better than they do for adult, HSC-driven leukemias. A good example is the modeling of childhood acute B-lymphoblastic leukemia (cALL) (Böiers et al., 2018), where developmentally restricted progenitors were differentiated, and compared to their *in vivo* counterparts. This allowed the identification of the leukemic cell of origin, a lympho-myeloid progenitor affected in its B-lymphoid differentiation potential by the RUNX1-ETV6 translocation, a founding mutation in cALL. This showcases the potential of pluripotent stem cells to reveal the ontogenic differences between progenitors, and their varying susceptibility to preleukemic initiation. More recently, the gene fusion ETO2-GLIS2, associated with acute megakaryoblastic leukemia (AMKL) cases, was introduced in a healthy iPSC line via zinc finger nucleases. Global recapitulation of AMKL transcriptional program and increased self-renewal of progenitors were observed, but some differences in ERG and GATA1 activity, major features of human AMKL, were present when compared to patient cells. Further investigation will clarify if this is the consequence of differences in ETO2-GLIS2 expression or in developmental stage of hematopoiesis (Bertuccio et al., 2020).

7.3. Pluripotent stem cells as robust models for drug screening

Disease modeling in iPSCs has been consistently matched with hypothesis-driven drug testing. Those efforts involved hematopoietic differentiation *in vitro,* colony forming assays, colony re-plating potential and signaling studies.

For classical MPNs, JAK inhibitors (ruxolitinib, fedratinib and CYT387) were tested for their effects on erythroid colonies (Ery-P), showing strong growth inhibition, albeit non-specific for $JAK2^{V617F}$ (Saliba et al., 2013; Ye et al., 2014). Similar findings were obtained by inhibiting downstream pathways, like mTOR, HSP90 and PI3K. In iPSCs harboring $CALR^{ins5}$, the effects of 3-hydroxyanagrelide, a metabolite of anagrelide (used in ET patients), were studied on erythroid and megakaryocytic differentiation in liquid culture. Only a decrease in megakaryocytic output was observed (Takei et al., 2018).

In CML, the archetype of MPN, undifferentiated iPSCs with the BCR-ABL fusion were resistant to the tyrosine kinase inhibitor (TKI) imatinib, a first-generation drug used for the treatment of CML patients. Conversely, the proliferation of iPSC-derived HSPCs was effectively inhibited by imatinib (Bedel et al., 2013; Carette et al., 2010; Kumano et al., 2012), with the immature progenitors being less sensitive than mature $CD45^{+}$ cells (Bedel et al., 2013; Kumano et al., 2012). A recent study in CML-iPSCs identified one marker, ADAM8, as overexpressed in TKI-resistant immature hematopoietic cells ($CD34^{+}CD38^{-}$cells), compared to TKI-sensitive ones ($CD34^{+}CD38^{+}$ cells). ADAM8 plays a role in leukocyte recruitment in terminally differentiated hematopoietic cells, and its metalloproteinase activity could be inhibited chemically (GM6001), restoring TKI sensitivity in previously resistant $CD34^{+}$ progenitors (Bedel et al., 2013). Finally, HSPCs from an iPSC line with complex genotype (*BCR-ABL* and $JAK2^{V617F}$) proved sensitive to imatinib and pimozide (a STAT5 inhibitor), suggesting a synergistic effect for the combination (Sloma et al., 2017).

The use of iPSCs is advantageous in the case of rare neoplasms, as there are no other models: in the case of 8p11 myeloid/lymphoid neoplasms with eosinophilia, the effectiveness of several multi-TKI inhibitors was evaluated using colony forming assays, and while CHIR258, PKC412 (midostaurin) and ponatinib (all FGFR inhibitors) decreased the CFU numbers in highly specific fashion, imatinib showed no effect (Yamamoto et al., 2015).

In CMML and JMML, a classical target to decrease granulomonocytic expansion is the RAS/MAPK pathway. A recent study used CMML-iPSCs to investigate the inhibition of RAS (with salirasib) and MAPK pathway (with PD0325901), but also the effects of liposome clodronate, a molecule known to deplete monocytes/macrophages. The authors confirmed the decrease in HSPC numbers and serial replating for all those treatments, supporting the use of clodronate for treating CMML and JMML patients (Taoka et al., 2018).

The use of MDS/AML iPSC modeling for drug testing was further highlighted by Kotini et al (Kotini et al., 2017) and Chang et al (Chang et al., 2018). Indeed, the treatment with a hypomethylating agent 5-Azacytidine restores differentiation of low-risk but inhibits the growth of high-risk MDS MDS-iPSC derived HSPCs. The treatment of MDS/AML-iPSC derived HSPCs with rigosertib, a RAS signaling pathway inhibitor, efficiently inhibited a $KRAS^{mut}$ subclone (Kotini et al., 2017). The MDS-iPSC derived HSPCs carrying $SRSF2^{mut}$ alone were shown to be sensitive to splicing inhibitor E7107 or splicing modulators Cpd-1, Cpd-2, Cpd-3 and those carrying del(7q) to niflumic acid (Chang et al., 2018).

For AML-iPSCs, two types of drugs were tested against KRAS- and MLL-mutated lines, targeting DOTL1 (EPZ-5676) and MEK (PD98059 and Trametinib) (Chao et al., 2017). EPZ-5676 showed a broad impact on colony formation and replating potential, while the MEK inhibitors were effective against $KRAS^{mut}$, but not $MLL^{mut}/KRAS^{wt}$ iPSCs-derived hematopoietic cells. Cytarabine treatment was more effective on $MLL^{mut}/KRAS^{mut}$ than $MLL^{mut}/KRAS^{wt}$ hematopoietic cells. This result is comparable with the clinical observation that the sensitive clone was present before cytarabine treatment, while undetected in the relapse sample. These experiments could therefore be exploited to predict the emergence of treatment resistance.

Finally, iPSC models have also been used to test drugs targeting inherited conditions. The SDS-iPSCs model showed that inhibition of TGF β signaling by SD208 improved the number of erythroid and myeloid progenitors, but only in absence of additional anomalies, like the del(7q) (Ruiz-Gutierrez et al., 2019). iPSCs with a *FANCA* truncating mutation (Liu et al., 2014) were used for testing approved compounds. Resveratrol (Sirt1 activator) had no impact on hematopoiesis, while danazol (an androgen) enhanced both FA- and control iPSC differentiation. More interestingly, doramapimod (p38 kinase inhibitor) and

Table 1
Drugs tested in iPSC models.

Disease	Mutated gene	Tested drugs	Drug Effect	Reference
Classical MPN	$JAK2^{V617F}$	Ruxolitinib, Fedratinib, CYT387 (JAK inhibitors) RAD001 (mTOR inhibitor) AUY922 (HSP90 inhibitor) LY294002 (PI3K inhibitor)	Dose-dependent inhibition of erythroblast progenitors, not specific for $JAK2^{V617F}$ mutation	(Saliba et al., 2013; Ye et al., 2014)
	*CALR*ins5	3-hydroxy anagrelide (platelet-lowering agent)	Inhibition of megakaryopoiesis, no effect on erythropoiesis	(Takei et al., 2018)
CML	*BCR-ABL* fusion	Imatinib (tyrosine kinase inhibitor)	Decreased growth of hematopoietic progenitors; no effect on undifferentiated iPSC; decreased susceptibility of immature leukemic cells	(Bedel et al., 2013; Carette et al., 2010; Kumano et al., 2012)
		GM6001 (metalloproteinase inhibitor)	Restored TKI sensitivity in previously resistant $CD34^{+}$ progenitors	(Miyauchi et al., 2018)
	BCR-ABL and $JAK2^{V617F}$	Pimozide (STAT5 inhibitor) + Imatinib	Decreased number of CFU, possible synergistic effect of the two drugs	(Sloma et al., 2017)
8p11 myeloproliferative syndrome	*FGFR1/CEP110* fusion transcript	CHIR258, PKC412 and ponatinib (FGFR inhibitors) Imatinib	Dose-dependent reduction of CFU number and of cell viability in liquid cultures No effect	(Yamamoto et al., 2015)
CMML	*EZH2, NRAS,* and *RUNX1*	PD0325901 (MEK inhibitor) Salirasib (Ras inhibitor) Liposomal chlorodronate (macrophage depleting agent)	Decreased number of CFU Decreased number of CFU and reduction of serial re-plating capacity	(Taoka et al., 2018)
AML	*MLL* rearrangements + *KRAS* mutation in a subclone Del(7q), complex karyotype + *MLL3* + *KRAS* mutation in a subclone	EPZ-5676 (DOTL1 inhibitor)	Dose-dependent inhibition of hematopoietic colony formation and replating potential; no impact on undifferentiated iPSC	(Chao et al., 2017) (Kotini et al., 2017)
		PD98059 and Trametinib (MEK inhibitors)	Dose-dependent inhibition of hematopoietic colony formation and replating potential (only for $KRAS^{mut}$ clone)	
		Cytarabine Rigosertib (Ras inhibitor)	Higher sensitivity of $MLL^{mut}/KRAS^{mut}$ hematopoietic cells compared to $MLL^{mut}/KRAS^{wt}$, implications for clonal relapse Higher sensitivity of hematopoietic progenitors in presence of $KRAS^{mut}$	
High-risk MDS	*t(1;7)(q10;p10)* + *Gata2* + *U2AF1* + *ETV6* ± *MYB*	5-Azacytidine	Inhibitory effect on the growth of hematopoietic progenitors	(Kotini et al., 2017)
Low-risk MDS	Del(7q) + *SRSF2* + *PHF6*	5-Azacytidine	Rescue of BFU-E and CFU-GEMM colonies	(Kotini et al., 2017)
	*SRSF2*Del (7q)	E7107 (splicing inhibitor)	Inhibitory effect on the growth of hematopoietic progenitors	(Chang et al., 2018)
		Cpd-1, Cpd-2 and Cpd-3 (splicing modulators)	Inhibitory effect on the growth of hematopoietic progenitors	(Chang et al., 2018)
		Niflumic acid	Inhibitory effect on the growth of hematopoietic progenitors	(Chang et al., 2018)
SDS	*SBDS*	SD208 (TGF-β receptor I inhibitor)	Increased number and size of erythroid and myeloid colonies, but only in absence of additional anomalies like del(7q)	(Ruiz-Gutierrez et al., 2019)
Fanconi anemia	*FANCA* truncating mutation	Resveratrol (Sirt1 activator)	No impact on hematopoietic differentiation	(Liu et al., 2014)
		Danazol (synthetic androgen)	Enhanced hematopoietic differentiation; not specific for FA-iPSCs	
		Doramapimod (p38 kinase inhibitor)	Improved hematopoietic differentiation, downregulation of the expression of inflammatory cytokines	
		Tremulacin (anti-inflammatory compound)		
SCN	*ELANE*	Sivelestat (NE-specific small molecule inhibitor)	Restored NE localization, promotion of promyelocyte survival and differentiation	(Nayak et al., 2015)
		MK0339 (NE inhibitor)	Improved cell survival and increased maturation of neutrophils	(Makaryan et al., 2017)

tremulacin (anti-inflammatory) partially rescued the altered hematopoiesis, improved $CD34^{+}CD43^{+}$ cell production and CFU-GM colony formation. This effect correlated with the suppression of inflammatory cytokine levels (IFNγ, TNFα, IL-6) at the transcript level, providing the first evidence of a positive effect of inflammation suppression on FA hematopoiesis.

These examples clearly demonstrate the value of iPSC models to test drugs, in a hypothesis-driven fashion. The effects of different drugs are summarized in Table 1. Further optimization of the differentiation protocols is required to use iPSCs in high-throughput unbiased drug screening, a much-sought after application due to their human pathology relevance (Papapetrou, 2016).

8. Conclusion

Since its discovery, iPSC technology has had a significant impact on our understanding of the pathophysiology of many inherited and acquired hematological disorders. Several teams demonstrated that iPSCs can contribute to drug development, as a powerful technological platform for drug discovery and repurposing efforts. The main advantage lies in the maintenance of the patient genetic make-up in iPSCs, paving the way for the personalized use of iPSC-derived cells for assessing drug efficacy and toxicity *ex vivo*. The powerful combination of genome editing and iPSCs promises that this technology will lead to new transformative treatments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

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