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Modeling myeloid malignancies with patient-derived iPSCs

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Modeling human diseases with patient-derived induced pluripotent stem cells (iPSCs) offers unique research opportunities and is particularly attractive for hematology research. Whereas monogenic inherited blood diseases featured prominently among the first proof-of-principle studies of iPSC modeling, malignant hematologic disorders have been off to a slower start. This has been due to challenges in the derivation of iPSCs from cancer cells and the need to establish robust differentiation protocols and to standardize phenotypic assays of iPSC-derived hematopoiesis. Recent studies of iPSC modeling of myeloid malignancies exploited the clonal heterogeneity of patient samples to derive genetically matched normal controls and recapitulate the clonal evolution of the disease. Comparisons of the malignant phenotypes and molecular signatures of primary leukemic cells, derived iPSCs, and their hematopoietic progeny stress the importance of the cell-of-origin in oncogenesis and enable investigation of the interplay between cell identity and the cancer genome. Larger collections of genetically diverse iPSC lines and more readily scalable hematopoietic differentiation protocols, ideally mimicking adult bone marrow-derived hematopoiesis, would further empower applications of iPSC modeling in myeloid malignancy in the future. Nevertheless, with recent progress in this field, the stage is set for the wider adoption of this model system by the hematology community.

The derivation of human induced pluripotent stem cells (iPSCs) in 2007 ushered in a new era in the modeling of human diseases, including those affecting the hematopoietic system [1–3]. Significant advances over the past decade have enabled investigators to increasingly incorporate iPSC models in their research. iPSCs can empower diverse research studies, ranging from investigations into basic disease mechanisms to more translational applications such as therapeutic target discovery, drug testing, compound screening, toxicity testing, and generation of cells for transplantation [2,4].

Whereas monogenic inherited blood diseases were among the first to be modeled with iPSCs [5], malignant hematologic disorders have been more challenging. The challenge primarily lay in the relative difficulty of generating iPSC lines from blood cancers and, secondarily, in the unavailability of established assays to assess phenotypes relevant to hematologic malignancies in hematopoietic cells derived from human pluripotent stem cells (hPSCs), including human embryonic stem cells and iPSCs.

Here, I only discuss the iPSC modeling of myeloid malignancies, including myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPNs), and acute myeloid leukemia (AML). Progress in modeling lymphoid diseases with iPSCs has been more limited, mostly due to the current unavailability of efficient differentiation protocols for deriving lymphoid lineages from hPSCs in vitro or in vivo. Due to space limitations, we will also not discuss iPSC models of familial predisposition to MDS/AML, which have been reviewed elsewhere [6,7].

A slow start

Unlike inherited genetic diseases, in which the disease-causing mutations are present in the germline and therefore passed on to all somatic cells, cancerous genetic lesions, whether gene mutations or karyotypic abnormalities, for the most part arise postnatally in somatic cells of the hematopoietic stem/progenitor cell compartment (HSPC) in the bone marrow. Therefore,

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whereas patient-derived iPSC models of inherited monogenic diseases can be derived through reprogramming any accessible cell type, modeling malignancies requires reprogramming cells of the hematopoietic lineage that are descendent from the malignant clone (Figure 1). In the case of myeloid malignancies and premalignant diseases, the latter reside in the stem/progenitor and more differentiated myeloid compartments, most often excluding the lymphoid lineage. The progenitor compartment appears to be the most efficient to reprogram [8], at least in part because it is the most proliferative because cell division is a well-documented requirement for reprogramming to pluripotency [9–11]. Therefore, most reprogramming efforts have used unfractionated mononuclear cells or sorted $CD34^+$ cells from the bone marrow (BM) or peripheral blood (PB) as the starting cells and prestimulated them in early cytokine medium. Others have used erythroblasts as the target cell type with seemingly comparable success [12,13].

A first challenge to the generation of iPSC lines from leukemia is the heterogeneity of the starting cell sample. The bone marrow and the blood typically contain a mixture of normal cells and cells derived from the premalignant or malignant clone and there are generally not markers available for their prospective isolation. Fortunately, a wealth of information on the genetic lesions found in myeloid malignancies has become available in recent years through large-scale sequencing of MDS and AML genomes [14,15]. These detailed catalogs of nearly all recurrent genetic lesions in myeloid cancers can now be leveraged to characterize in depth the cellular composition and clonal hierarchies of patient samples and to select the most appropriate starting specimens for reprogramming. Through mutational analysis by next-generation sequencing (either whole-exome sequencing or targeted gene panel sequencing) that includes information on variant allele frequencies (VAFs) and parallel evaluation of large-scale genetic abnormalities such as deletions and translocations with karyotyping and fluorescence in situ hybridization, the normal to malignant cell ratio can be quantitated and the presence of subclones inferred. It is important to obtain this information from the actual sample (or another frozen aliquot thereof) that is used for reprogramming and preferably immediately before the initiation of reprogramming because the clonal composition can vary between different samples from the same patient due to disease progression, remission, administered therapies, transplantation, or just sampling factors. This composition can also vary because prestimulation of cells in culture can skew the clonal ratios, most frequently by depleting malignant cells that generally survive poorly in ex vivo culture conditions.

Although the heterogeneity of the starting cells can be measured in advance of reprogramming and can even be turned into an advantage (more on this below), the biggest challenge in the derivation of myeloid malignancy iPSCs is oftentimes the relative refractoriness of malignant cells to reprogramming. This appears to be a general feature of malignant cells that is not limited to hematopoietic tissues and manifests as a preponderance of normal lines among the derived iPSCs, even in cases when the starting cell sample is overwhelmingly clonal [7,13,16–18]. The exact reasons for this are unclear, but they likely involve the aberrant genetics and potentially also epigenetic states of malignant and premalignant cells. Specific genetic lesions have been well documented to both enhance and impede reprogramming to pluripotency [7,13,19–24]. Another factor may be the degree of "pre-apoptotic priming" of MDS and AML cells, which is especially manifested in ex vivo conditions. Consistent with this idea, MPN cells, which, in

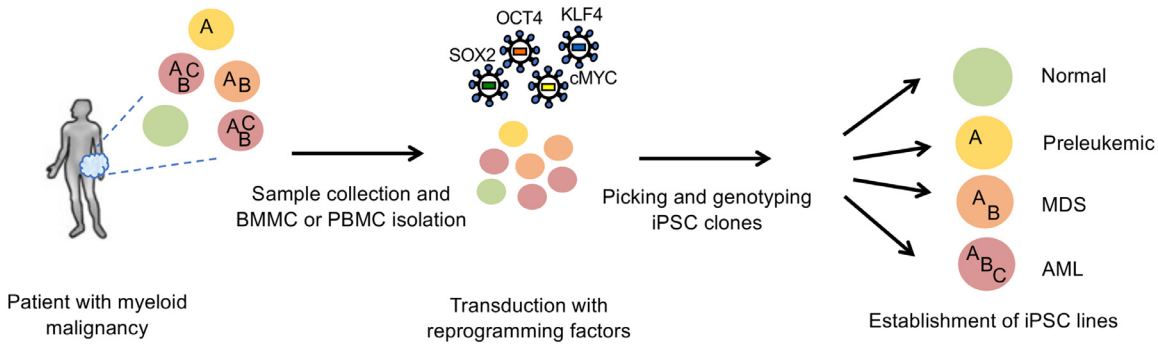

Figure 1. iPSCs capturing different disease stages, as well as normal iPSCs, can be generated from the same patient in one reprogramming experiment. Patient BM cells are genetically characterized to infer genetic composition. After transduction of either BM or PB mononuclear cells (BMMCs or PBMCs) with the Yamanaka reprogramming factors OCT4, SOX2, KLF4, and cMYC, clones with hPSC morphology are chosen, expanded, and genetically characterized. Clones with the desired genotypes are further expanded to establish iPSC lines. These can be indefinitely cultured and cryopreserved in an undifferentiated state. The scheme depicts an ideal case in which four distinct iPSC genotypes are captured in one reprogramming experiment. More often, these experiments yield between one and three different clones and subclones. A, B, and C depict different genetic lesions (mutations or larger-scale abnormalities such as chromosomal deletions or translocations).

contrast to MDS and AML cells, survive and proliferate well ex vivo, reprogram with high efficiency, comparable to that of normal cells [25–32].

A second challenge to iPSC modeling in malignant hematology until recently has been the lack of a framework of phenotypic and functional readouts of iPSC-derived hematopoiesis. Although iPSC lines can be maintained indefinitely in culture, they are fundamentally different from immortalized cell lines in that their self-renewal is attained through a pluripotent state and not oncogenic transformation [7]. They can thus only be propagated in an undifferentiated state akin to that of early embryonic cells. As will be further discussed below, it is now apparent that the pluripotent state is dominant over any disease-related state, including cancer, and that pluripotent stem cells maintain little, if any, phenotypic, transcriptional, or epigenetic characteristics of the somatic cell from which they came. Disease phenotypes, including malignant ones, manifest mainly or solely in the specific cell type in which the disease originates and can therefore only be read out following in vitro directed differentiation of the iPSCs to the appropriate cell type. The success of an iPSC disease model thus hinges upon a good differentiation protocol. Differentiation protocols generally involve the addition of morphogens and growth factors in a manner that requires temporal, dose, and sequence precision to guide cells through defined developmental decisions [33]. In turn, a good differentiation protocol critically depends on our current level of understanding of the ontogenesis of a specific tissue. Although we know quite a lot about the ontogeny of hematopoiesis compared with that of other tissues, many gaps in our knowledge remain and most of what we know is deduced from studies in the mouse, with uncertain equivalency to the human system. What is certain is that the hematopoietic cell populations derived from human iPSCs are distinct in their developmental stage and lineage potency from primary cells from adult bone marrow and, furthermore, are dependent on the specific differentiation protocol used to derive them. Several hematopoietic differentiation protocols have been developed and used by different groups of investigators, with dissimilar outputs. These include embryoid body-based and the spin-embryoid body variation thereof, co-culture with stromal cell lines (mainly the murine BM line OP9), and monolayer cultures [34,35,36–38]. Therefore, in order to identify hematopoietic disease phenotypes in disease iPSCs, we first need to characterize and contrast them to the hematopoiesis derived from normal hPSCs. To this end, we recently attempted to define a phenotypic road map of myeloid transformation by characterizing the hematopoiesis derived from a panel of iPSCs representing distinct stages during the progression of myeloid malignancy, as discussed in more detail below [13].

Overview of published studies

MPN

A few studies have reported iPSC models of MPN, both chronic myeloid leukemia (CML) and non-CML, the latter consisting primarily of the JAK2-V617F mutant MPN, as well as of the pediatric MDS/MPN overlap syndrome juvenile myelomonocytic leukemia [25–32,39–42]. In contrast to MDS and AML cells, MPN cells can be reprogrammed with efficiencies comparable to that of normal cells [25–32]. Most MPN modeling studies so far have focused on examining responses and resistance to clinically used kinase inhibitors, mainly JAK2 inhibitors and imatinib [25,27,31,32,43–45].

The finding in several studies that undifferentiated CML iPSCs do not show sensitivity to imatinib despite expression of BCR-ABL, unlike differentiated hematopoietic cells, which are sensitive, have prompted investigators to suggest the use of iPSC models to study drug resistance [25,27,32,44,45]. These observations and others that we will discuss below reflect the requirement of a particular cellular context for disease phenotypes and drug responses to manifest. This context likely consists of cell-type-specific gene expression and/or DNA and chromatin modifications. Along these lines, Amabile et al documented that, unsurprisingly, reprogramming erases methylation patterns of CML cells [42].

MDS

The first MDS-iPSC lines were derived from two patients harboring chromosome 7q deletions (del7q) [16]. Del7q is a characteristic chromosomal abnormality in MDS. Despite being one of the first recurrent karyotypic aberrations to be recognized in hematologic malignancy and the fact that it is known to be associated with adverse prognosis, how del7q contributes to MDS remains unclear. Most evidence pointed to haploinsufficiency of one or more genes residing on chr7q. Modeling large structural genetic abnormalities such as chromosomal deletions is impractical in the mouse or other model organisms because synteny is not well conserved. Indeed, homology for human chr7q is dispersed in four different mouse chromosomes. We thus modeled del7q in human iPSCs and, with a combination of genetic engineering of deletions of variable lengths and a phenotype rescue screen, we were able to first phenotypically map a minimal region of 20 Mb on chr7q and subsequently pinpoint candidate haploinsufficient genes residing in it. In a subsequent study, some of these iPSCs that, in addition to the del7q, also harbored a mutation in the splicing factor gene *SRSF2*, were used to investigate the effects of this recurrent mutation in the cellular phenotype and RNA splicing [46].

AML

Additional iPSC lines from patients with MDS and MDS that had progressed to AML (secondary AML) were subsequently generated [13]. Importantly, comprehensive genetic analysis (with whole-exome sequencing, gene panel next-generation sequencing, karyotyping, and fluorescence in situ hybridization) was performed to uncover all MDS- and AML-associated lesions in the starting BM or PB cells and derivative iPSC lines. The various derived iPSC lines could thus be assigned to the main clones, subclones, or normal cells, thus generating a panel of iPSCs capturing the entire spectrum of myeloid transformation from preleukemia to low-grade MDS, high-grade MDS, and secondary AML [13]. Upon hematopoietic differentiation of this collection of iPSC lines, phenotypes with disease-stage specificity or graded severity were identified and used to construct a phenotypic framework of the clonal evolution of MDS/AML. Chao et al. also derived iPSCs from two patients with AML with *MLL* translocations [47]. A striking and quite unexpected finding in both studies was that HSPCs derived from AML-iPSCs are able to serially engraft a lethal leukemia in immunodeficient mice. This is in stark contrast to hematopoietic cells derived from normal hPSCs (human embryonic stem cells and iPSCs) that notoriously fail to engraft long term [34,38,48–50]. Both of these studies also showed that cellular and molecular leukemic features are not manifest in the pluripotent state, but reappear upon hematopoietic differentiation [13,47]. Chao et al. documented this more thoroughly at the molecular level with DNA methylation and transcriptome analyses, showing that undifferentiated AML-iPSCs cluster together with normal iPSCs, indicating the similarity among all pluripotent cells, whereas AML-associated DNA methylation and gene expression changes are reestablished upon hematopoietic differentiation [47]. These findings are in agreement with a study of CML-iPSCs mentioned above, as well as other iPSC modeling studies of non-hematopoietic and non-malignant diseases, documenting that disease and normal iPSC lines cluster together at the undifferentiated state and are highly similar in gene expression. Furthermore, in both studies, AML-iPSCs showed potential for differentiation into non-hematopoietic tissues of all three embryonic germ layers in vitro and in vivo in teratomas without evidence of malignant features, highlighting the requirement of a specific cellular context for malignant features to appear.

Lessons learned

Despite the relatively small number of iPSC modeling studies conducted thus far, especially in MDS and AML, these have nevertheless yielded very important observations, some rather unexpected.

Lesson #1: Malignant cells are poor reprogrammers

Unlike the derivation of "classic" immortalized cell lines, which is only possible from fully malignant cells, malignant cells do not have an advantage in reprogramming to pluripotency. In contrast, they establish iPSC lines with generally lower efficiency than normal cells. Kotini et al. reported a success rate of 50% in deriving MDS and AML iPSCs (four out of eight patients), whereas an additional three of the other four samples gave only normal iPSC lines [13]. Although genetic alterations almost certainly influence the reprogramming efficiency (some possibly also positively), strong associations between specific genetic lesions and reprogramming potential have not emerged yet and will require additional studies using larger patient cohorts.

Lesson #2: Clonal heterogeneity is a good thing

The admixture of normal and clonal cells in the BM and PB of patients and their clonal heterogeneity can be leveraged to derive both disease and normal iPSC lines in the same reprogramming round and to derive iPSCs capturing different clones and subclones from distinct stages of the clonal evolution of the disease [13,16,31,47]. In some cases, reprogramming can also be used to clarify the clonal composition and/or the clonal hierarchies of the starting cell population. In many cases, the genetic analysis of the patient sample does not allow precise reconstruction of the clonal architecture. For example, in advanced disease, most mutations may appear clonal and the information on the order by which they were acquired is often lost. Because reprogramming is clonal, the retrospective analysis of derived iPSC lines can aid the deconvolution of the original sample and resolve the temporal order of acquisition of two or more mutations by identifying distinct clonal iPSCs with one, two, or more mutations. In another example, divergent clonal evolution can be revealed by iPSC reprogramming. Divergent acquisition of signaling mutations is not an uncommon late event in AML, but mutational analysis of bulk populations of patient cells does not allow discrimination of whether these mutations have independently arisen in different clones. By analyzing clonal iPSC lines, subclonal mutations can be unambiguously assigned to the same or different cells. The latter has been demonstrated in a study by Kotini et al., where two subclonal RAS pathway mutations detected in the starting sample (KRAS G12D at VAF 0.12 and NRAS Q61R at VAF 0.03) could be assigned to different subclones because they were found as single lesions in different iPSC clones [13].

Often, the derivation of normal iPSC lines from the same starting patient sample is not feasible, likely for reasons related to the initial cellular composition and

growth dynamics. In these cases, normal genetically matched iPSCs can be derived in separate reprogramming experiments from different cell populations such as T cells [47], bone marrow stromal cells [51], skin fibroblasts, or any other available cell type. An alternative or complementary approach is to derive isogenic "corrected" iPSC lines by gene editing of the disease lines, a practice that is becoming much more widespread with the advent of CRISPR technology [46,52,53]. Of course, this is not easy or even feasible for large or complex chromosomal alterations and copy number variants and is generally limited to creating isogenic pairs for simple gene mutations or, at best, single chromosomal abnormalities such as deletion of chromosome 7q and translocation t(9;11) involving the *MLL* gene [16,54]. In contrast, patient cell reprogramming in principle allows the modeling of any cancer genome. In addition to the chr7q deletions and *MLL* translocations mentioned above, complex karyotype AML, a rather frequent genetic subtype of AML, has been successfully reprogrammed [13].

Lesson #3: Pluripotency trumps the malignant phenotype

Several studies have now documented that, at the pluripotent state, iPSCs lose effectively all discernible features of malignancy even though they retain the oncogenic lesions and express their gene products. These include cellular behavior (iPSCs from myeloid malignancies can make non-hematopoietic cell types with seemingly normal morphology and function [47]), molecular signatures (gene expression, DNA methylation [42,47]), and oncogene dependence and drug responses (CML-iPSCs are resistant to imatinib at the pluripotent state [25,27,32,44,45]). This is presumably due to the widespread remodeling of chromatin that occurs upon reprogramming and the establishment of a robust and dominant pluripotent cell state. In contrast, differentiation of these iPSC lines to hematopoietic cells restores their malignant phenotypes [13,47]. These observations uniquely confirm and highlight an important principle in cancer research, which is that oncogenesis requires both the cancer genome and the appropriate cell lineage determined by a cell-type-specific transcriptional program and/or chromatin landscape.

Lesson #4: Line-to-line variation can and should be overcome

Line-to-line variation in phenotypes and other cellular and molecular properties has plagued the field of disease modeling with iPSCs, but this can and should be overcome with appropriate controls, sufficient replicates, and robust differentiation protocols; in other words, with good scientific practices and high-quality experiments. The first and perhaps most important step is awareness of the caveats. Although intraline and interline variation is not unique to the iPSC field, one should keep in mind that every iPSC line comes from a single cell of the starting population that may or may not be representative of the majority of the population and that propagation in culture can result in acquisition and propagation of genetic and chromosomal abnormalities that may change cellular properties. Although genetic variation has been shown to be, unsurprisingly, the main contributor of differences in differentiation potential and cellular phenotypes among iPSC lines [16,55–59], epigenetic differences, either preexisting or established during reprogramming, can also contribute to variable behavior among lines of the same genetic composition. Thanks to the work of several developmental hematology researchers, protocols for efficient and reproducible directed differentiation of hPSCs into hematopoietic lineages have vastly improved in recent years, enabling robust experiments. Therefore, disease-associated phenotypes have been uncovered in hematopoietic cells derived from iPSC models of myeloid malignancies, which include decreased MDS or increased AML proliferation in vitro, differentiation block or impaired differentiation potential into one or more myeloid lineages (MDS and AML), dysplastic morphological changes (MDS), increased cell death (MDS), and cytokine independence (JAK2-V617F MPN) [13,16,31,46]. Essential to establishing genotype-to-phenotype connections and to practically all iPSC modeling studies is the use of appropriate control lines. These could be, in order of decreasing strength, as follows: isogenic lines generated by gene editing with CRISPR or other systems to introduce a mutation associated with myeloid malignancy in normal iPSCs or correct it in patient-derived iPSCs, ideally including complementary pairs of isogenic lines; genetically matched disease and normal iPSC lines derived from the same patient (not strictly isogenic); normal iPSCs derived from related healthy donors (i.e. from a family member of the patient); and normal iPSCs derived from unrelated healthy individuals. In the latter case, it is essential that iPSC lines from a sufficiently large number of different individuals should be used and contrasted to lines from multiple patients to account for variation due to genetic background.

Lesson #5: AML-iPSC-HSPCs engraft!

The ability of HSPCs derived from AML-iPSCs to robustly and serially engraft in immunodeficient mice was a rather unexpected finding and is currently the only reported example of robust, high-level, long-term engraftment of hematopoietic cells derived from any type of hPSCs through directed differentiation alone (without the forced expression of transcription factors)

[13,47]. This engraftment potential is presumably endowed by the leukemia genome. AML-iPSCs may thus provide a unique opportunity and a valuable tool with which to investigate the engraftment requirements of hPSC-derived cells and apply this knowledge toward the generation of engraftable HSPCs from normal hPSCs without genetic modification.

Wish list for the next decade

iPSC models are particularly well suited to research in hematology. Hematopoietic cells are easily accessible, can be isolated relatively easily, and can often be grown short-term ex vivo. Importantly, myeloid malignancies are very well characterized genetically, which makes tracking the clonal relationships between patient cells and derivative iPSCs, as discussed earlier, a relatively straightforward task. Despite the significant advances, however, many more technical challenges and open questions remain. Therefore, the wish list for the next decade is as follows:

- Larger collections of iPSC lines capturing diverse genotypes of MPN, MDS, and AML need to be generated and become available to the scientific community in parallel with the dissemination of expertise in their maintenance and hematopoietic differentiation.
- More scalable differentiation protocols with which we can obtain large numbers of hematopoietic cells or, alternatively, robust ways to maintain hPSC-derived HSPCs as expandable cell lines [46,60,61], will enable additional experimental capabilities such as high-throughput genetic and small molecule screens.
- We need to better characterize the developmental stage of iPSC-derived hematopoiesis and, importantly, to understand the limitations it poses to modeling premalignant and malignant blood diseases that mostly originate in adult cells. Although hPSC-derived hematopoiesis likely corresponds more closely to fetal liver (FL)-derived than adult BM-derived hematopoiesis, there is no reason to not expect that it will recapitulate at least some disease-relevant cellular and molecular features because FL- and BM-derived HSPCs have much in common in terms of transcriptome and epigenetic landscape. Should protocols to derive adult-type hematopoiesis from hPSCs and, importantly, to derive engraftable hPSC-HSPCs become available, these will of course likely enable additional phenotypic and functional readouts.
- There is currently strong evidence that iPSC models capture disease-specific phenotypes, but these are limited to cell-autonomous disease features. The incorporation of microenvironmental components in these models in the future may expand the utility of iPSC models to investigate interactions of hematopoietic cells with their niche.
- Several studies have now established the usefulness of iPSC models to establish genotype-to-phenotype connections. However, epigenetic changes may also play a role in the pathogenesis of myeloid malignancies. Given the dramatic epigenetic changes that occur upon reprogramming to pluripotency, it is currently totally unknown and rather unlikely that iPSC-derived cells will retain any preexisting disease-associated epigenetic abnormalities. Current evidence suggests that reprogramming erases disease-associated transcriptional signatures and epigenetic marks and that those marks that are caused by an oncogenic genetic lesion (e.g. BCR-ABL) are reestablished in the hematopoietic progeny of these iPSCs [42,47]. Therefore, the expectation is that iPSC models of mutated epigenetic regulators (e.g., DNMT3A, TET2, ASXL1, EZH2) will capture abnormal marks upon hematopoietic differentiation. In contrast, any epigenetic lesions that may have been present in the primary cells that are not genetically determined (by mutation of a gene encoding a chromatin regulator) would likely be lost.
- Undoubtedly, the major strength of iPSCs is that they offer genetically precise and controlled models for mechanistic studies. In view of a few recent studies reporting promising findings on the ability of iPSC models to predict drug responses [43,46], one can envision the use of iPSCs in precision medicine with the generation of cell banks of iPSC lines representing the major mutations and genotypes of MPN, MDS, and AML and their use in drug testing and screening. In the future, the development of patient-specific iPSCs as personal avatars is also not unthinkable. A challenge to such personalized medicine approaches is aligning the experimental timelines needed to create an avatar with the timelines of clinical care of the patient. In AML patients, the time from remission to relapse should be in most cases sufficient (>3 months) to allow for a personal iPSC model to be put in place.

Modeling malignant hematologic diseases with patient-derived iPSCs is an exciting new field under development that holds much promise. Although real discoveries that will solidify the usefulness of iPSC models are still awaited, with the hematopoiesis and hematology community increasingly embracing this new model system, they likely lie just around the corner.

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