

Application of induced pluripotent stem cells to primary immunodeficiency diseases

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Primary immunodeficiency diseases (PIDs) are a heterogeneous group of rare immune disorders with genetic causes. Effective treatments using hematopoietic stem cells or pharmaceutical agents have been around for decades. However, for many patients, these treatment options are ineffective, partly because the rarity of these PIDs complicates the diagnosis and therapy. Induced pluripotent stem cells (iPSCs) offer a potential solution to these problems. The proliferative capacity of iPSCs allows for the preparation of a large, stable supply of hematopoietic cells with the same genome as the patient, allowing for new human cell models that can trace cellular abnormalities during the pathogenesis and lead to new drug discovery. PID models using patient iPSCs have been instrumental in identifying deviations in the development or function of several types of immune cells, revealing new molecular targets for experimental therapies. These models are only in their early stages and for the most part have recapitulated results from existing models using animals or primary cells. However, iPSC-based models are being used to study complex diseases of other organs, including those with multigenic causes, suggesting that advances in differentiation processes will expand iPSC-based models to complex PIDs as well. © 2019 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.

Primary immunodeficiency diseases (PIDs) describe a group of clinically and genetically heterogeneous disorders that afflict lymphoid and myeloid lineages. PIDs have been recognized for a century, but the first molecular cause was not reported until 1972, when scientists realized that a child with severe combined immune deficiency (SCID) completely lacked adenosine deaminase, eventually leading to enzyme replacement therapy [1,2]. There are now a number of therapeutic options for PIDs, including cytokine-based strategies, gene therapies, and hematopoietic stem cell (HSC) transplantation [3–5]. Mutations in more than 300 genes have been associated with PIDs, but estimates assume thousands more are still to be identified [6]. Although most categorized PIDs are monogenic, advanced diagnostics are revealing that multigenic factors and somatic mosaicisms are also contributing factors. The rates of PIDs vary, with frequencies approaching as low as one in a million, and approximately 20% of PID cases were originally reported in single patients [7,8]. Reduced

costs and increased speeds have made next-generation sequencing (NGS) the standard for PID diagnosis; however, a considerable number of patients with unknown genetic etiology cannot be diagnosed due to the rarity of patients or the nature of the mutation (e.g., deep intronic mutations) [9]. Furthermore, findings should be confirmed with cell assays, which can be difficult to establish using primary hematopoietic cells due to the patient's condition. The result is delayed diagnosis, which complicates treatment and worsens morbidity and mortality.

Induced pluripotent stem cells (iPSCs) provide a new model for studying these challenging PIDs. iPSCs are somatic cells reprogrammed to the pluripotent state, thus making it possible to prepare an indefinite number of cells for disease study even in circumstances when cell accessibility is difficult or only few patients are available. Importantly, these reprogrammed cells share the same genome of inaccessible primary cells. The result is new understanding of the disease pathogenesis and new molecular targets for treatment. Indeed, iPSC models have already been constructed for several PIDs, either affirming or challenging current theories about the molecular disorders contributing to the disease.

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PID modeling with iPSCs

PIDs are a wide class and primarily associated with mutations that disrupt the development or function of leukocytes, but also with inborn errors of metabolism [10]. Depending on the severity, patients can show vulnerability to a whole range of infections (e.g., *IL2RG* mutations in SCID) or in some cases to just a few viruses (e.g., *TLR3* mutations in herpes simplex encephalitis) [11]. Diagnostics include functional cell assays and genomic screenings. Complicating treatment is that any given PID can be heterogeneous and have mutations in dozens of genes without any specific set appearing causative [12]. Some patients are at extreme risk even from birth. The continuing reduction of NGS costs has made newborn screenings a standard option in suspected cases even when there is no family history [13]. Indeed, NGS has revealed that PIDs, which were once considered an exclusively Mendelian and monogenic family, are far more heterogeneous and include many multigenic and somatic factors [14]. However, despite these advances, several patients escape positive diagnosis. In these cases, when a PID is suspected but no clear genetic cause is found, autologous iPSCs should be considered. Using iPSCs from patients, scientists have gained new insights on how different cell types are affected by a PID and the developmental stage in which the disease phenotype emerges [15,16].

iPSCs describe the reprogramming of somatic cells into an embryonic stem cell (ESC)-like state. ESCs are pluripotent and can replicate indefinitely. They and, by extension, iPSCs can model the development of all three germ layers, including all stages of hematopoiesis [17]. Exploiting these features, researchers have induced iPSCs to differentiate into an assortment of hematopoietic cells [18]. The first iPSCs were

generated by exogenously expressing a master set of transcription factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) [19,20]. Since the original iPSC studies, this master set has been used to prepare iPSCs from multiple types of somatic cells from multiple types of species, confirming universality of the reprogramming mechanism.

Just as there are species differences in embryonic and hematopoietic development, there are species differences in ESCs and iPSCs (collectively, pluripotent stem cells, or PSCs), namely, the quality of the pluripotency. Human PSCs have primed pluripotency, whereas mouse PSCs have naïve pluripotency [21]. The different states describe the stability of the pluripotency and have implications on the creation of chimeric animals. Researchers have managed to reset primed human iPSCs to the naïve state, but the chimeric potential of these cells has never been confirmed and, unlike mouse PSCs, no naïve human PSCs have been generated without first acquiring the primed state [22,23]. The pluripotency state is considered a key determinant for modeling early development. Therefore, although hematopoietic cells have been successfully generated from human primed PSCs, the quality of the cells may be improved if naïve PSCs are the starting source.

Aside from the development potential of iPSCs, they provide a unique model that can recapitulate disease phenotypes in vitro. Within 2 years of the first human iPSC report, scientists confirmed that patient cells can be reprogrammed to iPSCs, which were used to study the disease pathogenesis [24,25]. Moreover, patient iPSCs are the basis for several clinical cell therapies and drug studies (Table 1). Not included in these lists is an iPSC model that identified a candidate neutrophil elastase inhibitor that could recover mature neutrophils

Table 1. iPSCs in the clinical setting

Disease	Type of Therapy	Status
iPSC-based cell therapies		
Age-related macular degeneration [73]	Autologous and allogeneic transplantation of iPSC-based product	<10 patients have received transplantation of retinal epithelial cells
Aplastic anemia with platelet refractoriness [64]	Autologous transfusion of iPSC-derived platelets	Single patient has been recruited; awaiting regulatory approval
Heart failure [74]	Allogeneic transplantation of iPSC-based product	Patient recruitment
Parkinson's disease [75]	Allogeneic transplantation of iPSC-based product	First operation was done in November 2018
Spinal cord therapy [76]	Allogeneic transplantation of iPSC-based product	Approval received from university board of ethics; approval from the Japan national regulatory body is expected
Drug trials based on iPSC patient models		
Amyotrophic lateral sclerosis [77]	Drug (ergobine)	Phase II
Fibrodysplasia ossificans progressiva [78]	Drug (rapamycin)	<20 patients have received the drug in double-blind study
Progressive supranuclear palsy [79]	Drug (BMS-986168)	Phase II
Spinal muscular atrophy [80]	Drug (RG7800)	Terminated after Phase I

from patients with severe congenital neutropenia (SCN) and who are unresponsive to alternative treatments [26]. Important from an industry perspective, some of these drug candidates have come not from drug discovery, but rather from drug repositioning, which is estimated to lower the cost and time to approval by one-third [27].

Accordingly, several patient iPSC models have been made to investigate blood diseases, including those causing bone marrow failure, anemia, and leukemia [28]. These studies have not yet led to clinical application, but they have revealed insights about which developmental stage the disease first manifests. For example, reprogramming cells from Fanconi anemia patients identified the disturbed differentiation capacity associated with the disease to occur as early as the hemoangiogenic progenitor stage, biasing cells to differentiate away from hematopoietic lineages and toward endothelial lineages [29]. Another study using iPSCs from Diamond–Blackfan anemia patients found that erythropoiesis could be enhanced by a small chemical that induced autophagy via ATG5, a molecule required for autophagosome formation [30]. Another SCN study found that the efficiency of neutrophil differentiation from patient iPSCs with a mutation in *HAX1* was 25% that of healthy donor cells [31]. Instead, the differentiation tended to arrest at the myeloid progenitor stage and showed a higher propensity for apoptosis, consistent with the abnormal granulopoiesis seen in the disease. The transduction of *HAX1* by lentivirus recovered the low neutrophil count.

Fortunately, we have had exceptional access to consenting patients suffering from rare PIDs, allowing us to reprogram their cells and build models to examine monocyte and macrophage development. The findings from these investigations have confirmed other models or revealed previously unknown molecular causes (see below for examples).

Mutations to *NOD2* are the cause of Blau syndrome, a congenital monogenic granulomatosis. *NOD2* is a receptor that upon binding to muramyl dipeptide (MDP) activates the nuclear factor-kappa beta (NF- κ B) pathway, upregulating cytokines and chemokines [32,33]. We reprogrammed cells from two *NOD2* patients with the R334W mutation. In one group of iPSCs, the mutation was corrected by gene editing. In addition, cells from a healthy donor were reprogrammed with the mutation knocked in. Assays revealed distinctive responses by iPSC-derived macrophages to interferon-gamma (IFN- γ) stimulation based on the mutation [34]. In all cases, IFN- γ stimulation upregulated *NOD2* expression, indicating a priming role. However, only in cells derived from mutant iPSCs did IFN- γ alone activate NF- κ B (Figure 1A). In the corrected case, MDP binding was required after the IFN- γ

priming signal, suggesting that targeting IFN- γ could have therapeutic benefit. These results were confirmed in primary patient macrophages.

Neonatal-onset multisystem inflammatory disease (NOMID), or chronic infantile neurologic, cutaneous and arthritis (CINCA), is the most clinically severe form of cryopyrin-associated periodic syndrome. The autoinflammation primarily affects the nerves, skin, and joints, and patients will develop sensory problems due to meningitis, rashes, and joint deformities. The majority of cases are associated with a mutation in the *NLRP3* gene, which causes excessive secretion of interleukin-1 beta (IL-1 β) by monocytes [35,36]. Dysfunction in the *NLRP3* inflammasome is associated with several metabolic diseases such as gout and obesity, and many clinical therapies target the excessive IL-1 β or the *NLRP3* complex [37].

However, a large minority of NOMID patients do not express a pathological mutation in *NLRP3*, instead showing somatic mosaicism. One such case was shown in a single Japanese patient with a mutation in *NLRP3* [38]. Interest in this gene has risen in recent years due to its discovered role in several autoinflammatory syndromes [39–41]. Fibroblasts from the NOMID patient were reprogrammed into iPSCs and then differentiated into monocytes. Consistent with NOMID symptoms, the iPSC-derived monocytes secreted IL-1 β without a secondary signal. Interestingly, activation absent the secondary signal was true of only some iPSC clones despite all being made from the same patient (Figure 1B). Whole-exome sequence analysis of the two clone types revealed a somatic mosaicism of the *NLRP3* mutation T177A as causative. Correcting the mutation by gene editing resulted in monocytes that behaved normally. Many NOMID patients are diagnosed without identifying causal mutations and somatic mutations can occur at frequencies below the detection limit of NGS systems, reiterating the benefits of iPSCs for rare PIDs [42].

Nakajo–Nishimura syndrome (NNS) is an autosomal disorder caused by mutations in *PSMB8*, which encodes for $\beta 5i$ protein, one of three subunits specialized for the immunoproteasome [43,44]. Point mutations in this gene are associated with phenotypes beyond autoimmune symptoms, including muscle atrophy and lipodystrophy [45,46]. NNS patients are susceptible to rashes and skin eruptions and are usually treated with corticosteroids, but this therapy does not ameliorate the lipodystrophy [47]. In NNS, the upregulation of proinflammatory factors is attributed to the p38 mitogen-activated protein kinase (MAPK) pathway. As expected, myeloid cells differentiated from patient iPSCs showed reduced immunoproteasome activity, higher production of reactive oxygen species, and increased production of IL-6, MCP-1, and IP-10, cytokines and chemokines that are associated with the

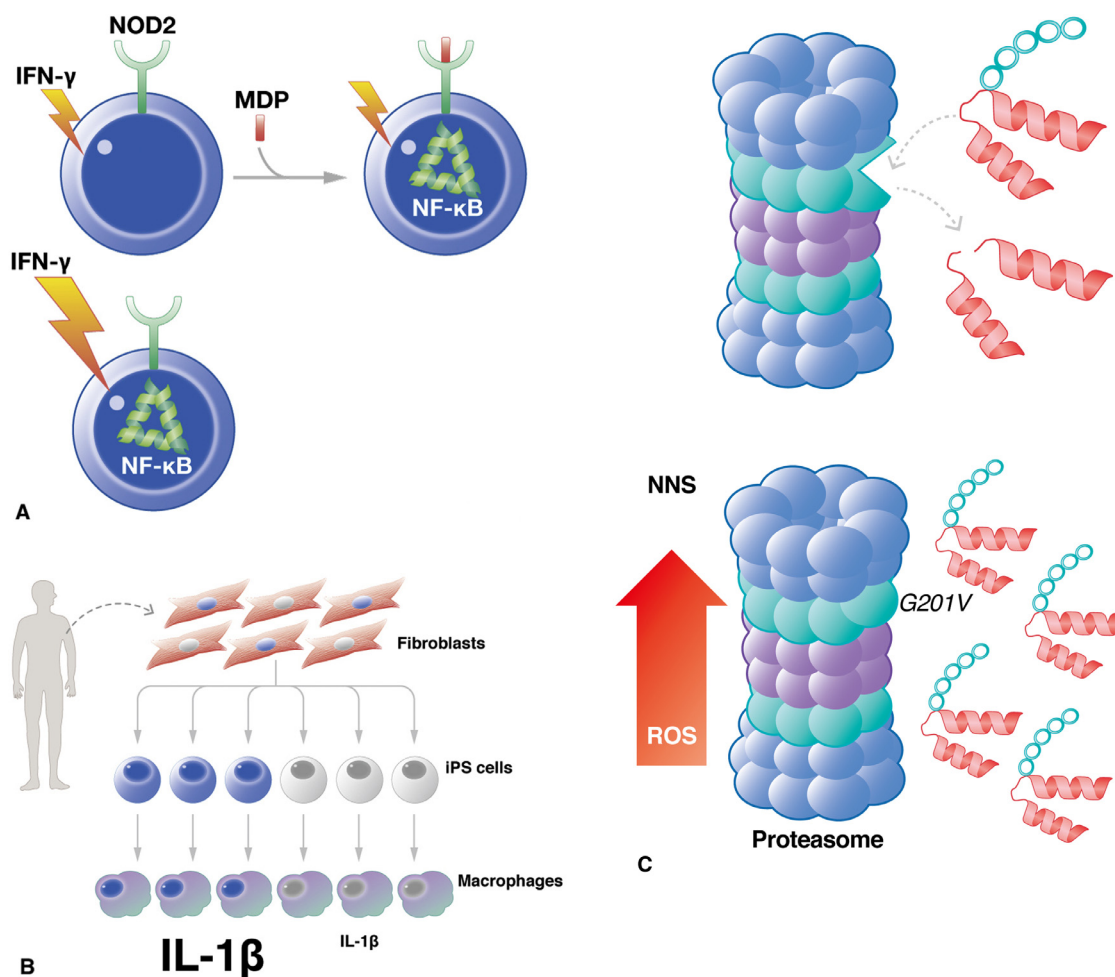


Figure 1. Patient iPSC models for PIDs. (A) Mutations in *NOD2* are a genetic cause of Blau syndrome. In normal conditions, IFN- γ acts as the priming signal in macrophages. The activating signal comes from MDP binding to the NOD2 receptor. Macrophages differentiated from patient iPSCs showed that IFN- γ acts as both the priming and activating signal, thus activating NF- κ B signaling even without MDP binding. (C) A point mutation in *PSMB8* (G201V) is the genetic cause of NNS. This mutation is believed to result in a structural change in the immunoproteasome. Myeloid cells differentiated from NNS-patient iPSCs showed reduced immunoproteasome activity. As a result, the myeloid cells produced excessive reactive oxygen species (ROS), causing the cells to secrete the inflammatory factors IL-6, MCP-1, and IP-10. The ribbon structures show processed proteins by normal immunoproteasome (top) and unprocessed proteins by mutant immunoproteasome (bottom), the latter causing ROS. (B) For most patients, the genetic cause of NOMID is attributed to a mutation in *NLRP3*. However, for some patients, no mutation in this gene exists. A NOMID iPSC model revealed that a subset of monocytes had a point mutation in *NLRP4* (cells with blue nuclei) and secreted IL-1 β without a secondary signal. Other monocytes without the *NLRP4* mutation (cells with grey nuclei) did not express this phenotype.

disease [48]. Furthermore, myeloid cells from patient and isogenic control iPSCs differed in that the mutant types showed evidence of being in a primed state even absent a stimulus (Figure 1C). Application of a p38 MAPK inhibitor to the cells suppressed the secretion of IL-6, MCP-1, and IP-10 in a dose-dependent manner, demonstrating how patient iPSCs in combination with gene editing for the generation of isogenic controls could contribute to drug discovery for PIDs.

Some PIDs associated with mutations in proteasome dysfunctions, like those seen with *PSMB8* mutations, are multigenic [49]. Such diseases have proven a degree more difficult to study with iPSCs. However,

success has been reported with complex disorders such as autism or idiopathic conditions such as amyotrophic lateral sclerosis, suggesting that a combination of iPSCs with other biotechnologies such as gene editing could lead to major breakthroughs for this category of PIDs [50,51]. Additionally, some PIDs are attributed to epigenetic alterations without any known genetic origin [52]. Cell reprogramming involves epigenetic resetting without changes to the genome and studies have shown how cell reprogramming in vivo can be used to study epigenetic causes in solid cancers [53]. Human iPSCs only recently reached their second decade and current disease models for PIDs could be viewed as a first

generation that complements existing cellular or animal models. Studies that build on models for other organs or with other technologies should advance the study of complex PIDs.

Generating monocytes and macrophages for disease study

Ideally, the differentiation of iPSCs to a specific cell type will follow the natural developmental program of the embryo. However, limited understanding of tissue development means that sometimes the induced cells do not recapitulate the function or maturity of primary cells, compromising the quality of the model. Most monocytes and macrophages develop from definitive hematopoiesis in the adult, although some tissue-resident macrophages such as microglia emerge from primitive hematopoiesis [54]. Macrophage subtypes can be acquired by coculturing the iPSCs with other cells in the tissue microenvironment [55]. Standard differentiation protocols for macrophages from iPSCs use a cocktail of cytokines, including IL-3 and macrophage-colony stimulating factor (M-CSF), to generate macrophages from hematopoietic progenitor cells (HPCs) [56,57]. IL-3 expands the HPC population with myeloid lineage bias, and M-CSF promotes terminal macrophage differentiation [58,59]. Although the generated cells are macrophages, they can show intermediate phenotypes of different subtypes, confounding their function and thus the accuracy of the corresponding cell model, reiterating that PID models still have room for improvement [60].

Few studies have investigated the engraftment potential of PSC-differentiated monocyte and macrophage progenitors in animal models and none to our knowledge have transplanted cells derived from PID patient iPSCs. The expression of specific sets of transcription factors that enhance the engraftment potential of progenitor cells that result in myeloid lineage has been reported. *HOXA9*, *ERG*, and *RORA* were found to induce the self-renewal and multipotency capacity of progenitors derived from human PSCs by reactivating HSC genes [61]. The additional expression of *SOX4* and *MYB* biased the progenitors toward erythroid and myeloid lineage upon engraftment in a mouse model. Building on that study, the same group showed that human PSCs could generate HPCs with good engraftment potential and reconstitute the myeloid and lymphoid in mice by overexpressing seven transcription factors (*ERG*, *HOXA5*, *HOX9*, *HOXA10*, *LCOR*, *RUNX1*, and *SPI1*), all of which have recognized roles in HSC development, maintenance, or lineage commitment [62]. Current PID iPSC models do not consider the expression of these factors in the differentiation protocol. Therefore, whereas the disease phenotype is expressed in cell assays, there is no

confirmation that these cells recapitulate the disease in animal models.

Furthermore, protocols that induce the myeloid differentiation of PSCs give comparably more consideration to the biochemistry (cytokines) than the biophysics (fluid dynamics) of the microenvironment. More than other tissues, blood cells operate in a very dynamic environment and consideration of the physical parameters in the culture system is expected to enhance the quality of the differentiated cells. In one example, megakaryocytes were induced from iPSCs by overexpressing *c-MYC*, *BM11*, and *BCL-XL* [63]. These megakaryocytes shed functional platelets, but their ploidy and low platelet output suggested that they were immature. Revision to a bioreactor that introduced both laminar flow and localized turbulent flow around the megakaryocytes resulted in a platelet count sufficient for transfusion therapy, and this project is now heading toward a clinical trial [64]. Currently, organoid technologies have arguably the best potential for recreating the chemical and physical microenvironment because they attempt to recapture the patterning and intermediate stages during natural development that lead to the final cell state [65].

Another factor affecting the quality of myeloid induction from PSCs may be the markers that we use to identify them. Typically, cell populations are defined by surface markers, but intracellular markers may prove more reliable, especially with regard to maturity or subtype. MicroRNAs (miRNAs) are noncoding RNAs that suppress translation by binding to complementary RNA and can be used as diagnostic tools for cancer and other diseases [66,67]. miRNAs have also been shown to regulate reprogramming and differentiation mechanisms, including those for myeloid and lymphoid lineages, and an analysis of miRNA expressions has indicated dynamic expressions of miRNAs during the differentiation of iPSCs to different hematopoietic cell stages [68–70]. Additionally, the overexpression of *SOX2* and *miR125b* was found to promote the direct differentiation of human fibroblasts into macrophage progenitors, which went on to produce mature macrophages when transplanted into mice [71]. These studies suggest that miRNAs could be useful markers for progenitors and that analyzing miRNA expression could contribute to the induction of higher quality hematopoietic cells. In response, colleagues of ours have developed the miRNA switch, an RNA-based synthetic structure that turns gene expressions on or off in living cells depending on the presence of specific miRNA or RNA-binding proteins (Figure 2). By constituting miRNA switches with complementary RNA that bind to intracellular markers for different cell types, they have successfully activated apoptotic genes to eliminate unwanted cells from a heterogeneous population, thus purifying the targeted cell subpopulations [72].

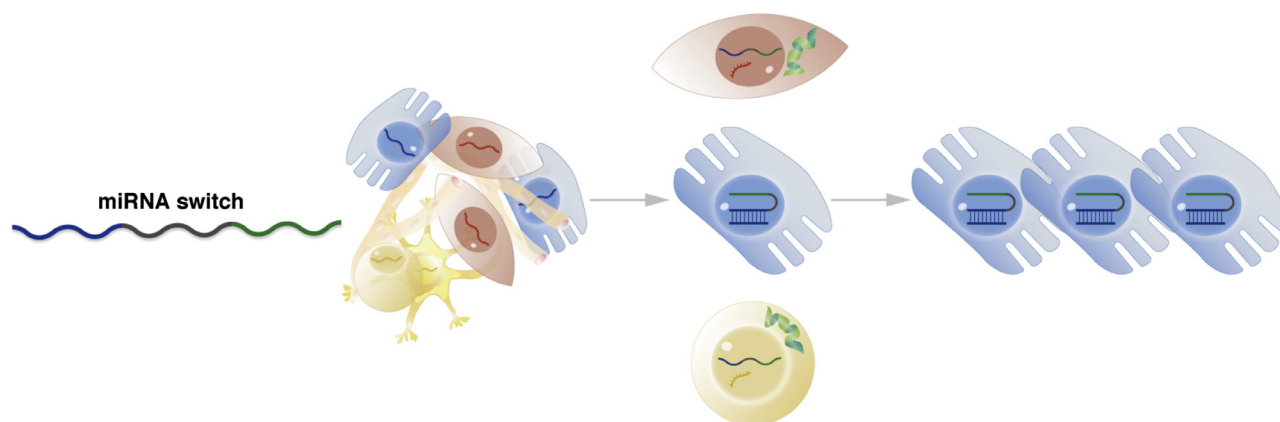


Figure 2. miRNA switches. miRNA switches use intracellular markers to turn genes on or off. In the schematic, the miRNA switch is a single-strand RNA that includes the sequence of an apoptotic gene (green) and a sequence (blue) complementary to miRNA that is highly expressed in blue cells but not in other cells. Binding of the miRNA to the complementary sequence causes a structural change in the mRNA that prevents the apoptotic protein from being expressed (green ribbon in yellow and red cells). Therefore, when this miRNA switch is transfected into the shown heterogeneous cell population, only the blue cells survive because their miRNA binds to the switch to inhibit protein synthesis (the loop in the RNA structure indicates a conformational change blocking translation). This approach allows for the selection of living cells with specific intracellular markers.

Conclusions

Advances in gene editing, NGS, and other technologies have made PIDs manageable, if not curable, for many patients. Scientists can now obtain unprecedented detail about the molecular and genetic causes, providing new targets for gene therapy and drug testing. However, despite similar symptoms, molecular causes frequently deviate in PID patients. In these cases, especially when patients are rare, assays of patient cells can be most informative, but sometimes primary cells are difficult to procure or simply are not informative. Accordingly, cell reprogramming, namely iPSCs, brings a revolutionary framework to the study of PIDs. Current models are still relatively simple and for the most part have confirmed other cell and animal models. However, as we clarify hematopoiesis and adjust differentiation protocols accordingly, patient iPSC models, which retain the genome of the primary cells, are expected to open the door to a new generation of cell and drug therapies.

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