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The Role of Induced Pluripotent Stem Cells in Research and Therapy of Primary Immunodeficiencies

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Abstract

The advent of reprogramming technology has greatly advanced the field of stem cell biology and nurtured our hope to create patient specific renewable stem cell sources. While the number of reports of disease specific induced pluripotent stem cells is continuously rising, the field becomes increasingly more aware that induced pluripotent stem cells are not as similar to embryonic stem cells as initially assumed. Our state of the art understanding of human induced pluripotent stem cells, their capacity, their limitations and their promise as it pertains to the study and treatment of primary immunodeficiencies, is the content of this review.

Introduction

Primary immunodeficiency diseases (PID) consist of a group of more than 150 mostly monogenetic conditions that predispose individuals to different sets of infections, allergy, autoimmunity and cancer [*1]. The study of human PIDs has allowed identification of genes that play a key role in immune system development and function. In several cases,

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identification of gene defects in patients with PID has preceded development of corresponding animal models. Furthermore, although humans and mice with defects in orthologue genes often share a similar phenotype, in some cases significant differences have emerged, thus limiting the significance of animal models to dissect the pathophysiology of human PIDs. Therefore, use of human samples remains a fundamental tool to study mechanisms of disease. For example, patient-derived $CD34^+$ hematopoietic stem cells (HSCs) can be used to study in vitro the differentiation of T and B lymphocytes and of myeloid cells in patients with various forms of severe combined immunodeficiency (SCID), congenital agammaglobulinemia or severe congenital neutropenia. However, the rarity of these conditions limits access to patient-derived HSCs and thus represents a considerable obstacle for mechanistic studies of disease pathophysiology.

Significant progress has been made in the treatment of human PIDs. For example, SCID is fatal within the first years of life but can be cured by means of hematopoietic cell transplantation (HCT) and- in selected cases - gene or enzyme replacement therapy. Initial gene therapy trials for X-SCID provided proof of principle that the underlying genetic defect could be overcome by transduction of autologous patient $CD34^+$ HSCs with a gamma retroviral vector carrying the γc gene, allowing long-term robust immune reconstitution [2]. However, the development of leukemia as a severe adverse effect made clear that the challenge to cure SCID had not yet been conquered. Integration of the vector close to a proto-oncogene, *LMO2*, led to insertional mutagenesis and clonal expansion of T cells in 5 of 20 patients with X-SCID treated in Paris and London [3,4]. Similar serious adverse events have been observed also after gene therapy for chronic granulomatous disease [5] and Wiskott-Aldrich syndrome [6]. This prompted development of novel and hopefully safer approaches to gene therapy, based on the use of self-inactivating retroviral and lentiviral vectors in which the LTR have been removed and the transgene is expressed of an internal promoter of lesser potency [7]. Nonetheless, none of these approaches will completely eliminate the risk of insertional mutagenesis. An alternative strategy is represented by locus-specific targeting and gene correction, based on homologous recombination (HR) with use of a correct repair matrix. Although the frequency of HR is low in somatic cells (10^{-6}), it can be significantly increased by using locus-specific nucleases such as zinc-finger nucleases (ZFN), meganucleases, and Transcription Activator-Like Effector Nucleases (TALENs), that introduce targeted DNA double strand breaks. Ultimately, the preclinical assessment of efficacy of this approach will rely upon use of patient-derived cells and demonstration of gene correction and functional reconstitution. Once again, the rarity of PIDs (and limited access to HSCs in particular) may represent a significant hurdle, unless new tools prove capable of providing viable alternatives.

Induced pluripotent stem cells (iPSCs) are a novel and practical tool for human disease modeling and correction, and in theory could serve as a limitless stem cell source for patient specific cellular therapies.

Initially envisioned as an equivalent to embryonic stem cells (ESCs), iPSC have now been recognized to bear more profound differences to ES cells than originally assumed. Reports of genetic instability of iPSCs have raised concerns about their potential use of in the clinical setting.

Taking a closer look at induced pluripotent stem cells, carefully evaluating their capacity and recognizing their limitations will enable us to accurately judge their potential and prevent us from prematurely dismissing their application in regenerative medicine.

Induced pluripotent stem cells

Pluripotency refers to the ability of stem cells to grow indefinitely in culture while maintaining the potential to give rise to any of the three germ layers. When Thomson et al first described the isolation of human ESCs from the inner cell mass of a blastocyst in 1998 [8], the impact that this breakthrough would have on medicine became immediately evident. The ethical dilemma surrounding the generation of human ESC lines and the desire to create patient specific stem cells was the driving force behind the quest of finding alternative ways to generate pluripotent stem cells.

Somatic cells can be reprogrammed to a stem-cell like state by transferring their nuclear contents into oocytes [9] or by fusion with ESCs [10], indicating that unfertilized eggs and ESCs contain factors that can confer pluripotency to somatic cells. Takahashi and Yamanaka hypothesized that the factors that play important roles in the maintenance of ES cell identity also play pivotal roles in the induction of pluripotency in somatic cells [11]. A screen of 24 candidate genes lead to the triumphant description of a tetrade of transcription factors, Oct4, Sox2, Klf4, and cMyc, sufficient to reprogram tailtip fibroblasts of mice into iPSCs. Reprogramming has since been successfully applied to a multitude of human tissues. A number of different strategies are currently being employed to deliver the reprogramming factors to the cell of interest. Integrating vectors (retroviral, lentiviral, inducible lentiviral), non-integrating vectors (adenoviral, plasmid) and DNA-free systems (modified mRNA, MicroRNA, Protein, Sendai virus) have all been successfully used to generate iPSCs, although with different efficiency [**12]. Integrating vectors may have a higher risk of insertional mutagenesis, but they may be designed to allow excision of the reprogramming transgene, while leaving a traceable footprint behind [13]. Codon-optimization of the transgenes has been shown to significantly increase efficiency of the reprogramming process [13]. RNA and protein based reprogramming systems come at lower efficiency and higher cost but are potentially preferable for therapeutic applications, because of lower safety concerns [**12].

In recent years, the scientific community has strived to adhere to consistent standards for the identification and functional assessment of iPSCs [14]. These include: (1) distinct morphologic characteristics; (2) unlimited capacity to self renew; (3) gene expression profiles that are comparable to ESCs (OCT4, SOX2, Nanog) with silencing of transgenes; (4) protein-level expression of key pluripotency factors (e.g., Oct4, Nanog) and ESC-specific surface antigens (e.g., SSEA-1 in mouse; SSEA-3/-4, Tra-1-60/-81 in human); (5) Karyotyping; (6) Functional assessment. Tetraploid complementation (direct generation of entirely ESC/iPSC-derived organisms) is the gold standard to establish pluripotency of murine iPSCs [15], while teratoma formation, demonstrating differentiation into elements of all three germ layers, serves as a surrogate in human iPSCs [**12].

Though similar, it is important to recognize that iPSCs are not the same as ESCs. When comparing bona fide human iPSCs derived from different tissue sources, incomplete erasure of tissue-specific methylation and aberrant *de novo* methylation lead to distinct genome-wide DNA methylation profiles and differentiation potential. Residual DNA methylation signatures characteristic of their somatic tissue of origin, may favor differentiation along lineages related to the donor cell, while restricting alternative cell fates [*16]. In contrast, the methylation pattern and differentiation potential of nuclear-transfer-derived pluripotent stem cells are more similar to classical embryonic stem cells than iPSCs are [*17].

Such ‘epigenetic memory’ of the donor tissue needs to be considered and may influence our efforts at directed differentiation of iPSC for disease modeling or treatment [**12]. Strategies to alter the cells epigenetic memory are topic of active investigation and include

use of histone-deacetylase-inhibitors, methyl-transferase-inhibitors, 5-azacytidine, and effectors of the Wnt signalling pathway.

For the study and treatment of primary immunodeficiencies, the implementation of robust and reliable methods for hematopoietic differentiation is of paramount importance. In the differentiation pathway to hematopoietic progenitors, pluripotent stem cells go through mesoderm cell lineage commitment. Three different methods have been employed to induce pluripotent stem cells to become mesodermal cells: (1) the formation of embryoid bodies (EB), (2) the co-culturing of pluripotent stem cells with stromal layers, and (3) the culturing of pluripotent stem cells on extracellular matrix proteins. The former two methods are mainly utilized to differentiate iPSCs into hematopoietic progenitors [18].

EBs are formed in suspension culture from iPSCs and contain elements of all three germ layers. EB derived erythroid and myeloid lineage precursors can be grown into colonies on methylcellulose. Stromal layers support iPSC colonies similarly to feeder cells. The most used stromal cell line is the OP9, which was established from an osteopetrotic (*op/op*) mouse, deficient in macrophage colony-stimulating factor (M-CSF), as M-CSF was shown to have deleterious effects on early development of hematopoietic cells (HPCs) [19]. Choi et al. have reported on the successful induction of iPSCs into myeloid progenitors by culturing iPSCs on OP9 cells with subsequent expansion and directed differentiation into neutrophils, eosinophils, dendritic cells, Langerhans cells, macrophages and osteoclasts [20].

OP9 cells also support lymphopoiesis [19]. The in vitro generation of cells of the adaptive immune system remains the most promising, yet the most challenging aspect of hematopoietic differentiation from iPSCs and is focus of active investigation.

Studying T cell differentiation in vitro

An important model for generating and studying T cell development in vitro makes use of fetal thymic organ cultures (FTOCs) [21]. Embryonic day E14 or E15 mouse thymic lobes are isolated and endogenous thymocytes are depleted using 2-deoxyguanoise or radiation. The thymus can then be seeded with human hematopoietic progenitors, human progenitor thymocytes, bone marrow derived progenitor cells, or umbilical cord blood cells. FTOCs are then cultured to allow for the generation of mature single positive (SP) T cells. It is perhaps not surprising that the FTOC system works well when culturing progenitors originating from the mouse, but is less efficient when human progenitors are used [22]. However, this also suggested that the human thymic stromal cells are more suitable for differentiating human progenitors towards SP T cells.

T cell development has been shown to depend on Notch signaling, which is required to instruct cells towards a T cell - rather than B cell – fate. Notch-1 conditionally deficient mice exhibit a block in T cell development, resulting in B cells in the thymus [23]. The expression of the Notch ligand Delta-like-1 (Dll1) in the OP9 cell line allows for T cell development to occur in vitro in the absence of the thymus for both mouse and human cells [24].

Using iPSCs and the OP9-DL co-culture system to generate human T lymphocytes

Several different strategies have been engaged in order to direct ESC and iPSC towards the blood lineage. Typically these approaches consist of either making embryoid bodies (EBs) from the ESCs or iPSCs and directing the EBs to differentiate towards the hematopoietic lineage, using a stromal co-culture approach, or even using serum and feeder free conditions

[25,26]. Thus, human ESCs and iPSCs have been used to generate cells with hematopoietic potential [26,27]. Generation of the erythrocyte lineage has been reported using human ESCs [28] and iPSCs [27,29–31]. Platelets [32] and neutrophils [**33,34–36] have also been successfully differentiated from hESCs or iPSCs. However, generation of other blood cells has been more limited. Human ESCs have been successfully used to generate dendritic cells [37,38], B cells [39], NK cells [40], and T cells [41–43]. Two recent studies show generation of B [*44] and NK [45] cells from iPSCs. Given the recent work with human pluripotent stem cells, and the previous work with mouse pluripotent stem cells, a feasible experimental approach is outlined in Figure 1. Until recently, the modeling of human blood diseases using patient-specific iPSCs was limited to polycythemia vera [46], sickle cell disease [*47,*48], dyskeratosis congenita [49], adenosine deaminase deficiency [50] and X-linked chronic granulomatous disease [**33]. The iPSC/OP9-DL system could allow for both human T cell development and human PIDs to be studied in greater detail *in vitro*.

Development of PID-specific iPSCs

Since the first successful report of a human iPSC line from a patient with ADA by Park et al [50], many more iPSC from patients with various forms of PID have been generated (Table 1) [33,50,*51].

Our laboratory has generated a repository of human iPSCs from a wide variety of fibroblast lines from patients with PID (Table 1 and Figure 2). This repository serves as a platform to model molecular mechanisms underlying immune and extra-immune manifestations of PID and to study various gene targeting approaches for disease correction.

Zou et al. have shown that iPSCs derived from patients with X-linked CGD (due to mutations of the *CYBB* gene) may be differentiated into neutrophils and recapitulate the disease phenotype, with failure to generate reactive oxygen species upon activation [**33]. Furthermore, these CGD-specific iPSCs may also serve as a platform to investigate novel approaches to gene correction. In particular, targeted introduction of the *CYBB* cDNA into a genomic “safe harbor” by means of ZFNs (delivered through a lentivirus) restored the ability of the patient-derived iPSCs to support generation of functional neutrophils [**33]. Similarly, correction of neutrophil function has been reported upon lentrivirus-mediated gene transfer using iPSCs from a mouse model of CGD [52].

The appeal of using iPSCs to model non-hematopoietic manifestations of primary immunodeficiencies may not be underestimated. For example, iPSC-derived neural progenitor cells may be used to investigate the molecular and cellular basis of central nervous system-restricted susceptibility to HSV-1 infection in patients with Herpes Simplex Encephalitis due to defects of the TLR3 pathway (Lafaille et al., submitted). Furthermore, the rapid development of tissue specific differentiation protocols might help us find pathology in organs that were previously thought to be unaffected by disease [53].

Induced pluripotent stem cells may also represent a new and powerful platform for preclinical testing of novel approaches to treatment, including gene correction. Gene repair “in situ” may be achieved using homing endonucleases [54], Zinc-finger nucleases [55] or Transcription Activator-Like Effector Nucleases (TALENs) [*56]. All these approaches employ nucleases that introduce targeted double stranded DNA breaks to increase significantly the rate of homologous recombination. Targeted genome modification can get even more sophisticated: in addition to correcting a mutation, selectable markers can be inserted simultaneously [*57]. This allows tracing cells that have undergone gene targeting and could prove an invaluable tool in the follow up of cellular therapies.

Slowly, the application of iPSCs for high throughput drug testing is gaining more attention. Generation of iPSCs from subjects of various ethnicity, followed by targeted differentiation into a wide range of primary human tissues, may represent a powerful and cost-effective strategy to assess how genetic and epigenetic differences influence the effects of small molecules.

The role that iPSCs could play in the treatment of degenerative conditions was recognized early on. More refined and more applicable to the treatment of PID is the use of iPSCs for patient specific cellular therapies. The basic concept is simple: iPSC are derived from readily available patient tissue and disease-causing mutations are corrected -preferable by site-specific gene targeting- at the pluripotency state. In vitro differentiation shall yield hematopoietic stem cells or more mature effector cells which can be screened at a single cell level for genetic integrity. Only cells that passed the molecular screen get selectively expanded before they are returned to the patient. (Figure 1).

Conclusions

The theoretical approach is easy to appreciate, but reality brings us back to the ground. A number of not insignificant obstacles have yet to be overcome before iPSC technology will make its way to the patient's bed. Hanna et al. masterly provided proof of principle in a humanized sickle cell anemia mouse model, that the sickle phenotype can be rescued by transplantation with gene corrected hematopoietic progenitors obtained in vitro from autologous iPSCs [*58]. But mice are not humans and what has been demonstrated in mice, the isolation and characterization of an engraftable hematopoietic stem cell, has not been achieved in the human setting so far. HSCs derived from human iPSCs or ESCs may display the characteristic markers of ex-vivo isolated HSCs, yet when transplanted into animals they fail to induce hematopoietic reconstitution [59]. Pioneering work from John Dick's group has helped to define the demarcation between human HSCs and multipotent progenitors (MPPs) with a goal of characterizing HSCs capable of long-term engraftment for stem cell-based therapies [60,61]. It is hoped that these studies may lead to identification of an ESC/iPSC-derived cell with in vivo engraftment potential.

Even if this hurdle is cleared, the question remains, how safe iPSC-derived cells will ultimately be. Substantial progress has been made in deriving iPSCs in which the oncogenic reprogramming factors are no longer present. A more subtle issue, however, is whether the reprogramming process itself creates an epigenetic state that predisposes any progeny of these cells to transform into cancer. Reports of karyotype instability [*62], accumulation of point mutations [*62], copy number variations of pluripotency and cell proliferation genes [*63] have begun to cloud the promise iPSCs once held for their use in the clinical setting. Last but not least, the question of immunogenicity of autologous iPSC has recently been raised [**64]. The statement made by Orkin and Motulsky's in their 1995 NIH review on gene therapy, that over 100 trials have failed to produce any beneficial effects, and that is paramount that clinical studies be designed to yield useful basic information irrespective of the clinical outcome is more relevant than ever.

The hymn of praise that the research community once sang on induced pluripotent stem cells has acquired a somber pitch. Nonetheless, the promise that reprogramming technology brought to the field of stem cell biology still exists. Delineation of the subtle but crucial differences that define iPSCs versus ESCs, HSCs versus MPPs will only be a matter of time. The concern that iPSC offspring gone astray could do substantial harm in the clinical arena is not without cause. What exactly the future of iPSCs will bring is hard to predict but is rather naïve to expect that their entry into the clinical realm will be smooth and without complications. The big quest is that researchers adhere to stringent principles of ethical

conduct and reproducibility of data. Only if we do so, we can anticipate the future with excitement rather than fear.

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differentiated from iPSCs can induce T-cell-dependent immune response in syngeneic recipients, and may thus pose a novel and unexpected hurdle toward potential clinical use of iPSCs. [PubMed: 21572395]

Highlights

- Reprogramming technology allows to generate induced pluripotent stem cells (iPSC) as a renewable stem cell source for use in research
- Disease modeling with iPSCs overcomes the limitations of available study material in the research of primary immunodeficiencies and serves as a platform to test gene correction strategies
- Robust in vitro hematopoietic differentiation protocols are of paramount importance to study the molecular mechanisms underlying immune manifestations of PID
- Human T lineage development can be modeled using the IPSC/OP-9DL system
- Safety measures need to be in place before translating iPSC technology into the clinical arena

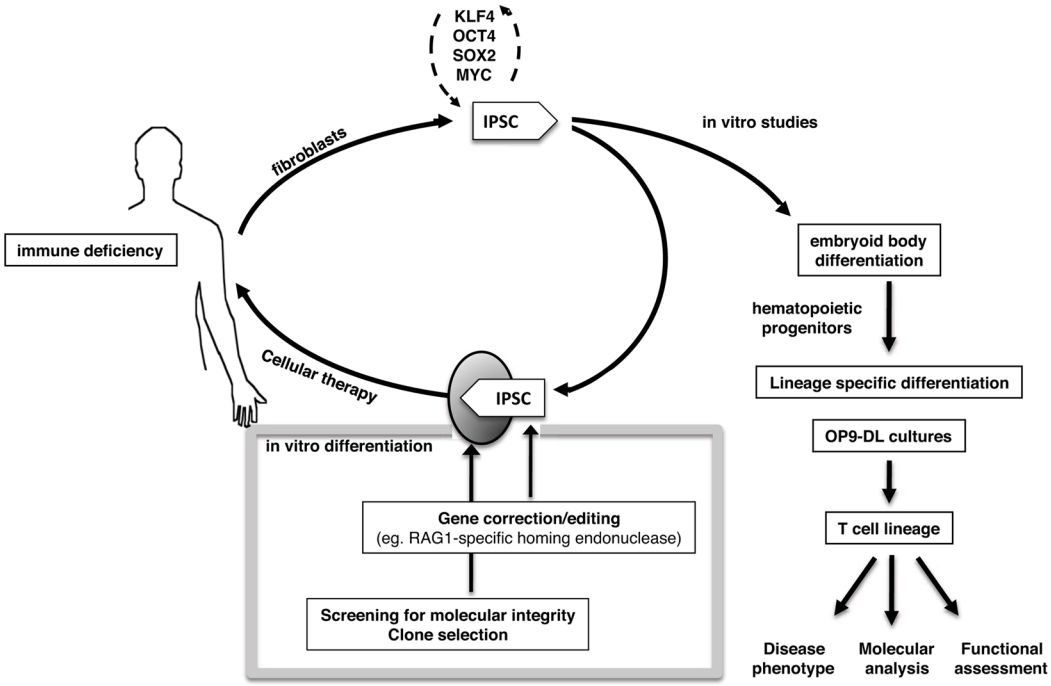

Figure 1.
Schematic representation of use of patient-derived iPSCs for modeling and correction of human immunodeficiencies.

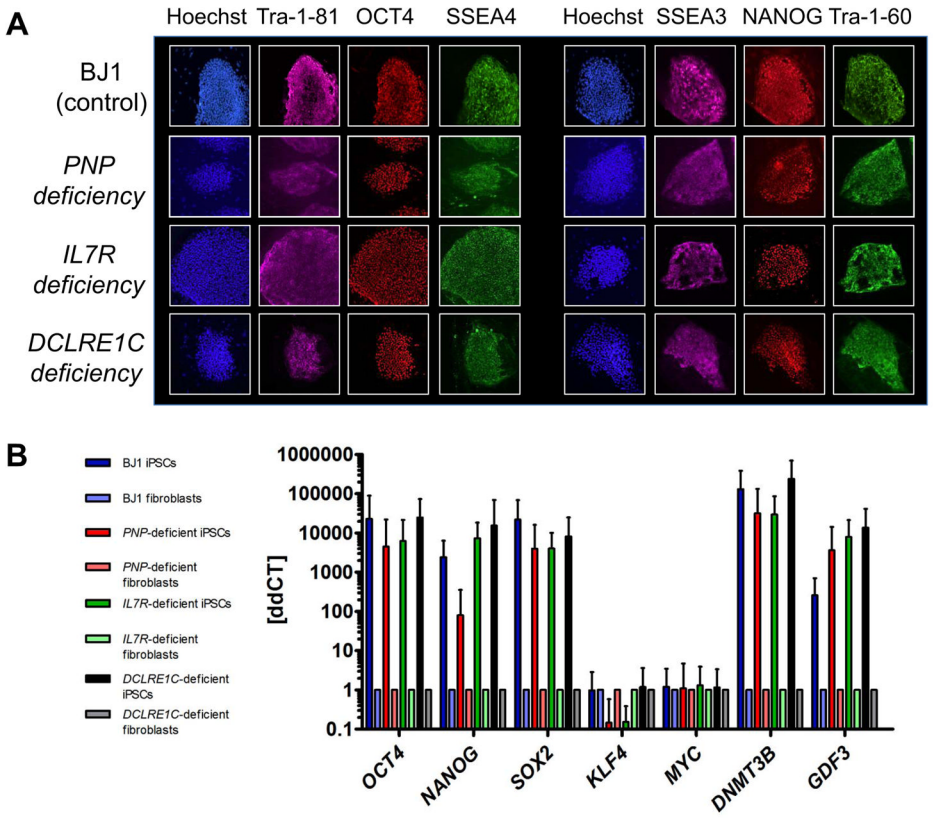

Figure 2. Characterization of the stemness and pluripotency profile of induced pluripotent stem cells (iPSCs) derived from patients with severe forms of immunodeficiency

Panel A): Immunofluorescence showing expression of markers of stemness (Tra-1-81, OCT4, SSEA4, SSEA3, NANOG, Tra-1-60) in iPSC lines derived from a healthy control (BJ1) and from patients with defects of purine nucleoside phosphorylase (*PNP*), interleukin-7 receptor (*IL7R*) and Artemis (*DCLRE1C*) genes. Panel B): quantitative-real time (qRT)-PCR analysis comparing expression of pluripotency genes (*OCT4, NANOG, SOX2, DNMT3B, GDF3*) in control- and patient-specific fibroblasts and in their derivative iPSCs. Relative expression levels were normalized to levels in fibroblasts. Similar levels of expression in fibroblasts and iPSCs are demonstrated for *SOX2* and *KLF4*, indicating silencing of the reprogramming transgenes.

Table 1

List of iPSCs obtained from patients with primary immunodeficiency diseases

Disease	Gene	Mutation	Disease model application	Reference
SCID	*ADA*	646G>A, exon 7; del nt1050–54 (GAAGA), exon 10	Lymphoid/myeloid Neuronal	Park et al. [50]; available through Coriell cell repositories
SCID	*RAG1*	c.1228C>T; c.2332C>T	Lymphoid	Pessach et al. [*51]
SCID	*IL7R*	c.221+2T>G; c.221+2T>G	Lymphoid	Unpublished data, this manuscript
SCID	*PNP*	c.383A>G, p.128D>G; c.383A>G, p.128D>G	Lymphoid Neuronal	Unpublished data, this manuscript
SCID	*DCLRE1C*	del exon1–4; del exon 1–4	Lymphoid, neuronal	Unpublished data, this manuscript
Leaky SCID	*RAG1*	c.1180C>T; c.1180C>T	Lymphoid	Pessach et al. [*51]
Omenn Syndrome	*RAG1*	c.256–257del; c.2164G>A	Lymphoid	Pessach et al. [*51]
Herpes Simplex Encephalitis	*STAT1*	c.1928_1929 insA; c.1928_1929 insA	CNS cell types	Pessach et al. [*51]
Herpes Simplex Encephalitis	*TLR3*	c.1660C>T; c.2236G>T	CNS cell types	Pessach et al. [*51]
Cartilage Hair Hypoplasia	*RMRP*	c. 27G>A; c. 27G>A	Lymphoid, myeloid, epithelial	Pessach et al. [*51]
Chronic Granulomatous Disease	*CYBB*	x. 458T>G; exon 5	Myeloid	Zou et al. [**33]

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