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Induced Pluripotent Stem Cells: A novel frontier in the study of human primary immunodeficiencies

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Abstract

Background—The novel ability to epigenetically reprogram somatic cells into induced pluripotent stem cells through the exogenous expression of transcription promises to revolutionize the study of human diseases.

Objective—Here we report on the generation of 25 induced pluripotent stem cell lines from 6 patients with various forms of Primary Immunodeficiencies, affecting adaptive and/or innate immunity.

Methods—Patients' dermal fibroblasts were reprogrammed by expression of four transcription factors, *OCT4, SOX2, KLF4,* and *c-MYC* using a single excisable polycistronic lentiviral vector.

Results—Induced pluripotent stem cells derived from patients with primary immunodeficiencies show a stemness profile that is comparable to that observed in human embryonic stem cells. Following *in vitro* differentiation into embryoid bodies, pluripotency of the patient-derived indiced pluripotent stem cells lines was demonstrated by expression of genes characteristic of each of the three embryonic layers. We have confirmed the patient-specific origin of the induced pluripotent stem cell lines, and ascertained maintenance of karyotypic integrity.

Conclusion—By providing a limitless source of diseased stem cells that can be differentiated into various cell types in vitro, the repository of induced pluripotent stem cell lines from patients with primary immunodeficiencies represents a unique resource to investigate the pathophysiology of hematopoietic and extra-hematopoietic manifestations of these diseases, and may assist in the development of novel therapeutic approaches based on gene correction.

Keywords

Primary Immunodeficiency; Induced Pluripotent Stem Cells; Reprogramming

Introduction

Primary immunodeficiencies (PIDs) comprise over 150 distinct disorders of immune system development and/or function[1]. Dissection of the cellular pathophysiology of PIDs has been largely based on *in vitro* studies using patient-derived cells and on analysis of suitable animal models. While largely successful, both of these approaches have important inherent limitations. In particular, many forms of PID are rare, severe and affect predominantly infants and young children. In these cases, access to biological specimens from affected patients may be problematic. Furthermore, there is significant heterogeneity of clinical and immunological phenotype among patients with different mutations in the same gene, but limited information is available on this diversity at the cellular level[2]. Finally, studies that aim to define the cellular pathophysiology of human PIDs are usually performed on blood samples, occasionally on the bone marrow, rarely on lymphoid tissues (thymus, lymph nodes, spleen) and almost never on non hematopoietic tissues, yet many forms of PID also include extra-immune manifestations[1, 3]. This is the case for immunodeficiency syndromes characterized by multi-system developmental defects (such as DiGeorge syndrome[4] and cartilage hair hypoplasia[5]), broad expression of the disease-specific gene (as in defects of DNA repair[6], NEMO deficiency[7], hyper-IgE syndrome due to STAT3 deficiency[8, 9], and adenosine deaminase deficiency[10]) or tissue-specific susceptibility to infections (as in herpes simplex encephalitis[11–13]).

On the other hand, while murine models of PID have provided key insights, they also carry significant inherent limitations because of differences in immune system development and function between mice and humans and the relative lack of phenotypic variability and heterogeneity of mutations in murine models as compared to PIDs in humans.

Following the demonstration in 2006 by Takahashi and Yamanaka that mouse fibroblasts can be reprogrammed into induced pluripotent stem cells (iPSC) through transient forced expression of defined transcription factors[14], generation of iPSCs from terminally differentiated human cells has been recently reported[15–17]. Similar to embryonic stem cells, these cells hold the unique potential to differentiate into various tissue cell types, including neurons[18–25], cardiomyocytes[26–28], hepatic cells[29–31], gastrointestinal cells[32], thymic epithelial cells[33], hematopoietic cells[34, 35] and many others[36–41]. Furthermore, iPSCs have also been used to correct genetic disorders in mice following gene targeting and homologous recombination[42, 43].

Over the last ten years, we have established an extended repository of fibroblast cell lines from patients with various forms of PIDs. This repository is also representative of the diversity of the clinical and immunological phenotype that is associated with different mutations occurring in the same gene. Using this collection of fibroblast cell lines, we now report on the successful generation and characterization of a series of PID-specific iPSCs that may serve as the foundation for future studies of disease pathophysiology and gene correction.

Materials and methods

Patients

Dermal fibroblast samples were obtained from 6 PID patients carrying mutations in different genes as detailed in Table I. Informed consent was obtained from a parent or a guardian. Study protocols were approved by Children's Hospital Boston Institutional Review Board.

Cell lines and culture

A previously reported human iPSC line [17], obtained by reprogramming dermal fibroblasts with retroviral vectors encoding SOX2, OCT4, KLF4 and c-MYC transcription factors, was used as an internal control.

Patient and healthy control fibroblasts were maintained in DMEM (high glucose and L-glutamine) media containing 10% FBS, 1 mM L-glutamine and penicillin/streptomycin (hFib media).

iPSCs were maintained in human embryonic stem (hES) cells medium, composed of DMEM/F12 (Invitrogen, Carlsbad, CA) containing 20% KOSR (Invitrogen), 10 ng/ml basic Fibroblast Growth Factor (bFGF, Gemini Bio-Products, West Sacramento, CA), 1 mM L-glutamine, 100 μM non-essential amino acids, 100 μM 2-β-mercaptoethanol, and penicillin/streptomycin. The cells were co-cultured on CF1 irradiated mouse embryonic fibroblasts (iMEFs, Globalstem Inc, Rockville, MD) as previously described[16, 17]. Expansion and splitting of the iPSC colonies was done by either mechanical passage or by the use of collagenase, as previously described[44].

iPSC differentiation into embryoid bodies (EB) was achieved by transferring iPSC colonies into low-adhesion plates free of feeder cells and using a bFGF-free hES medium as previously described[16, 17, 45].

Lentiviral reprogramming vector production

Lentiviruses containing the polycistronic lentiviral vector STEMCCA–LoxP previously described[46–49] were produced using a five-plasmid transfection system as previously described [50].

Reprogramming of fibroblasts and human iPSC generation

Fibroblasts were infected with the lentiviral reprogramming vector in hFib media supplemented with 5 μg/ml protamine sulphate (Sigma) for 24 hours. After 72 hours, cells were transferred onto iMEFs in hES media. iPSCs colonies with ES-like morphology started to appear after 3 to 5 weeks. Colonies were picked, sub-cloned and expanded by mechanical transfer into new plates containing fresh and adhered iMEFs, as previously described[16]. Several clones were derived and characterized from each fibroblast line (Table E1 in the Online Repository).

Immunohistochemistry

iPSCs colonies were stained for OCT4, NANOG, TRA-1-60, TRA-1-81, SSEA3 and SSEA4 as previously described[45]. Images were acquired with a Pathway 435 bioimager equipped with a 10x objective (BD Biosciences, San Jose, CA).

Quantitative real-time PCR (qPCR)

mirVana™ RNA isolation kit (Ambion) was used for total RNA extraction and reverse transcription preformed using qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD), according to manufacturer's instructions.

Real-time qPCR in PowerSYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) was performed on an AB 7500 Real-Time PCR system (Applied Biosystems). Primer sequences used for amplifying *OCT4, SOX2, NANOG, REX1, GDF3, hTERT, KLF4, cMYC, RUNX, AFP*, *GATA4*, *Brachyury*, *NESTIN*, *NCAM* and *β-ACTIN* were as previously described[16, 17, 45]. Results were normalized to β-actin *(ACTB)* gene expression and relative expression calculated by the ddCT method relative to expression levels in the individual parent cell lines using SDSv1 Software.

Mutation Analysis

Genomic DNA was isolated from dermal fibroblasts and iPSCs using the QiAMP DNA Kit (Qiagen, Valencia, CA). Genes known to be mutated in the patients were amplified by PCR as previously described, using primer sets as detailed in Table E2 in the Online Repository. Amplified products were purified using QIAquick PCR purification kit (Qiagen) and sequenced by the DF/HCC DNA Sequencing Facility. Sequences were analyzed using the Sequencher 4.8 software (Gene Codes Corporation).

In some of the compound heterozygote patients, PCR products were cloned using the TOPO TA Cloning® Kit with pCR®2.1-TOPO® vector (Invitrogen). Cloning products were amplified in competent bacteria, purified (QiaPrep miniprep kit, Qiagen) and later sequenced as described above.

Karyotype analysis

Karyotyping and G-banding were performed as described (see: Supplementary Methods in the Online Repository) in a blinded fashion by Cell Line Genetics, Madison, WI.

Results

We have established a repository of dermal fibroblast cell lines from more than 60 patients with various forms of PID, that are representative of defects in various components of the immune system. This repository of PID-specific fibroblast lines has been used to establish a pipeline for the systematic production of PID-specific iPSCs.

The initial cohort of iPSCs was derived from, TLR3 deficiency), immune deficiency associated with systemic disorders (cartilage hair hypoplasia, CHH), and early defects in T and B cell development (*RAG1* mutations) (Table I). For the latter, we sought to derive iPSCs from patients with a different clinical and immunological phenotype (SCID, leaky SCID, Omenn syndrome) associated with null or hypomorphic mutations in the same gene (*RAG1*). In parallel, iPSCs were also derived from healthy control fibroblasts.

Various strategies have been previously described for reprogramming somatic cells to pluripotency. We have made use of an excisable, human stem cell cassette (STEMCCA)-containing single polycistronic lentiviral vector, that allows transduction of four reprogramming factors, *OCT4*, *SOX2*, *KLF4*, and *c-MYC* (Figure E1 in the Online Repository). Following infection with the STEMCCA lentivirus, patient- and control-derived fibroblasts were maintained under stringent human ES cell supporting culture conditions as previously described[16, 17, 45]. After 3–5 weeks of culture, ES-like colonies emerged and were picked and expanded. Several clones were derived from each of the fibroblast line (Table E1 in the Online Repository).

Human iPSC colonies were initially selected based on similarity to hES colonies, with discrete and compact colony morphology (Figure 1, left column). The selected colonies were then expanded and studied for expression of stemness markers, including Tra-1–81, Tra-1–60, OCT4, NANOG, SSEA3, and SSEA4. As shown in Figure 1, all iPSC colonies demonstrated uniform expression of these pluripotency markers.

Expression of pluripotency-related genes was also evaluated by quantitative RT-PCR. As compared to the parental fibroblast lines, patient- (and control-) derived iPSCs demonstrated robust expression of pluripotency-associated genes such as *OCT4*, *SOX2*, *NANOG*, *REX1*, *GDF3*, and *hTERT* (Figure 2). As an internal control of pluripotency gene expression profile, we used a previously reported control iPSC line[17] that had been generated using 4 retroviral vectors, each of which contained one of the four reprogramming factors. A similar profile of gene expression was demonstrated in the newly generated patient- and control-derived iPSCs and in reference iPSCs.

To confirm pluripotency and the ability to support multilineage differentiation, the PID-specific iPSC lines were allowed to differentiate *in vitro* into embryoid bodies as previously described[17, 45]. Similar to what was previously shown for hES, tight clusters of differentiating cells formed by day 7, and later cavitated, becoming cystic, by day 10 (Figure 3). Both PID-specific and control iPSCs showed expression of markers of all three embryonic germ layers (ectoderm, mesoderm, and endoderm) (Figure 3), thus confirming their ability to develop along multiple lineages.

To confirm patient-specific origin, we analyzed each of the iPSCs and the parental fibroblast lines for the specific gene mutation(s) identified in each patient. In all cases, genetic identity was observed between patient-derived fibroblasts and iPSCs (Figure 4A).

It has been previously reported that reprogramming of somatic cells to pluripotency and prolonged culture of hES cells may result in clonal somatic chromosomal aberrations[47, 51, 52]. We tested representative PID-specific and control-derived iPSC lines for karyotypic integrity. At least one line was analyzed for each patient-specific iPSCs (Table E1 in the Online Repository). For each line assayed, cytogenic analysis was performed on 20 G-banded metaphases. All but one of the various PID-specific iPSC lines that were analyzed demonstrated normal karyotype (Figure 4B). In one of the iPSC lines derived from a patient with RAG1-deficient SCID, 2 cells out of the 20 analyzed carried a trisomy of chromosome 7 (47,XX +7) and hence may represent a clonal chromosomal aberration. Two other lines derived from the same patient demonstrated a normal karyotype.

Discussion

The field of iPSCs is rapidly growing as novel reprogramming strategies and protocols that allow differentiation of iPSC into various cell types become available. In spite of this progress, variable efficiency of the nuclear reprogramming process, incomplete maintenance of iPSC stemness profile, failure to achieve transgene silencing, and integration-dependent effects on endogenous genes expression remain significant challenges[53–55].

To generate iPSCs from patients with various forms of PID, we have chosen to use a single lentiviral vector expressing the four common reprogramming factors *OCT4, SOX2, KLF4,* and *c-MYC* that are coded on a single polycistronic cassette. Using this vector, we have succeeded in generating multiple iPSC clones for each of the fibroblast cell lines that we have infected. A very high efficiency of reprogramming had been also observed with a murine version of the STEMCCA cassette[48]. Another potential advantage of this vector is that the STEMCCA cassette is flanked by two inverted LoxP sites, thus permitting excision of the lentiviral sequences by transient expression of the Cre recombinase[46, 47]. This strategy limits the residual genetic signature that is left following the reprogramming process to a minimum, and removes all reprogramming transgenes.

We have demonstrated that the PID-specific iPSCs generated with this approach exhibit a robust stemness gene expression profile. Furthermore, iPSC lines expressed TRA-1-60, a marker that has been shown to identify fully reprogrammed cells, capable of pluripotency[50]. In keeping with this, when cultured under appropriate conditions and allowed to differentiate into EB, iPSC lines expressed genes specific for each of the three embryonic layers. Nuclear reprogramming is thought to result from transient expression of the *OCT4, SOX2, KLF4,* and *c-MYC* transgenes, followed by induction of endogenous genes while the transgenes are silenced. Comparison of the pattern of expression of *OCT4, SOX2, KLF4,* and *c-MYC* genes in our series of PID-specific iPSCs is consistent with this notion. In particular, *KLF4* and *c-MYC* were expressed at similar levels in patient-derived iPSCs, their parental fibroblasts, and in reference iPSC cells, whereas iPSCs maintained high levels of expression of *SOX2* and *OCT4*. Since transcription of all four transgenes contained in the STEMCCA cassette is under control by the same promoter, these data suggest silencing of the transgenes, and differential induction of the endogenous genes. This has been recently confirmed using a modified version of the STEMCCA lentiviral vector also containing an m-Cherry reporter gene[46].

Clonal chromosomal aberrations have been previously reported in aged hES cells[51, 52]. Maintenance of karyotypic integrity is an important feature, when considering using patient-derived iPSCs to study the pathophysiology of the disease at the cellular level. With one single exception, all PID-specific iPSC lines tested retained a normal karyotype, demonstrating that genomic integrity is generally maintained at this level of resolution after reprogramming. However, assessment of genomic integrity remains an important test that should be performed on all newly generated iPSCs.

Prior to this study, generation of iPSCs had been reported only for one type of PID, namely ADA deficiency[45], using four retroviral vectors to allow transduction of the reprogramming factors. We have now shown that iPSCs can be generated with high efficiency from patients who suffer from various forms of PID that affect different arms of the immune system. However, it is possible that some forms of PID remain resistant to reprogramming. In particular, use of integrating vectors might fail to induce reprogramming in fibroblasts from patients with defects in DNA repair, because of toxicity and cell death associated with insertion of the vector. In this case, alternative strategies could be considered to generate

iPSCs, such as delivery of the reprogramming factors through non integrating vectors, or transient correction of the cellular defect.

Generation of a repository of iPSCs from patients with various forms of PID provides unique research opportunities (Figure 5). In vitro generation of T lymphocytes has already been reported for hES cells cultured on stromal OP9-DL4 cells[56–58]. If a similar approach becomes available for human iPSCs, it would be possible to compare the cell-intrinsic potential of iPSCs carrying different mutations in the same gene to support T cell differentiation. For example, use of the three patient-derived iPSC lines with mutations in the *RAG1* gene but with differing clinical phenotypes could provide a previously unforeseen experimental avenue to directly compare the efficiency and fidelity of human thymopoiesis.

Patient-derived iPSCs may also represent a unique tool to investigate in greater detail the pathophysiology of extra-hematopoietic manifestations associated with PIDs. Indeed, we have been able to differentiate iPSCs derived from patients with genetically-determined susceptibility to herpes simplex encephalitis into various mature cell types of the central nervous system, and by this mean dissect the cellular and molecular phenotype of the disease (Lafaille and Pessach *et al*., submitted). Similarly, it may become possible to study differentiation and function of thymic epithelial cells and heart cells starting from iPSCs from patients with DiGeorge syndrome, or inflammatory responses in various cell types obtained from iPSCs derived from patients with NEMO deficiency. Finally, iPSCs can be used as a limitless source of stem cells in which novel strategies to achieve gene correction may be tested. In particular, they share with ES cells a higher susceptibility to homologous recombination and thus represent a promising tool to study the ability of zinc-finger nucleases, meganucleases and sleeping-beauty transposons to mediate gene repair[54,55].

In conclusion, we have reported on the successful generation and characterization of iPSCs from patients with various clinical PID phenotypes and underlying genotypes.

The iPSCs technology is still at its early days. Limitations and potential pitfalls of this approach include, among others, variability in the efficiency and validity of the reprogramming strategy, and the possible introduction of genomic abnormalities that may lead to increased tumorigenesis. These problems must be addressed before considering use of these cells in clinical settings. Nevertheless, it can be anticipated that this novel technology will provide new insights into the pathophysiology of PIDs, and facilitate development of novel and more effective forms of treatment for these disorders.

Clinical Implications

Induced pluripotent stem cells derived from patients with primary immunodeficiencies represent a unique resource to study the pathophysiology and to develop novel therapeutic approaches for these disorders.

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Abbreviations

bFGF	basic fibroblast growth factor
CHH	cartilage hair hypoplasia
HSE	herpes simplex encephalitis
iMEFs	irradiated mouse embryonic fibroblasts
iPSC	induced pluripotent stem cells
OS	Omenn syndrome
PID	primary immunodeficiency
SCID	severe combined immune deficiency

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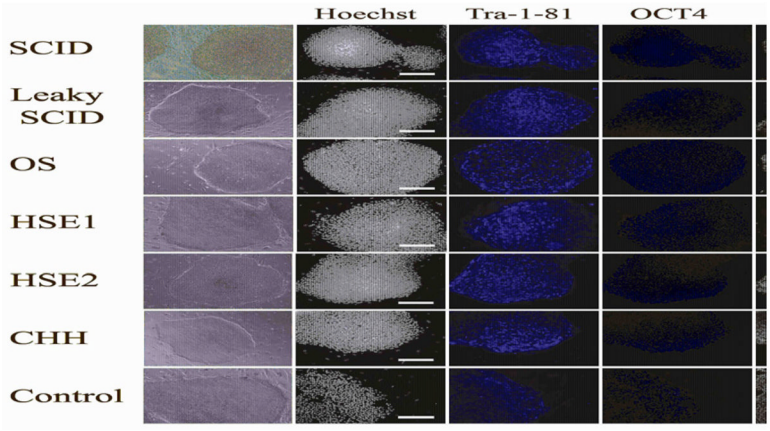

Figure 1. PID-specific iPSC express markers of pluripotency

PID-specific iPSC have morphology similar to hES cells (left column) when grown in coculture with iMEFs and express pluripotency markers including Tra-1–81, OCT4, SSEA4, SSEA3, NANOG and Tra-1-60 as shown demonstrated by immunohistochemistry. Nuclear staining with Hoechst 33342 is shown in the second and sixth columns to indicate the total cell content per image.

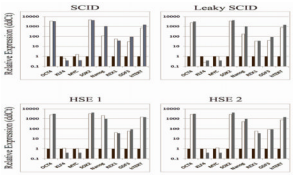

Figure 2. PID-specific iPSC show a gene expression panel similar to reference iPSCs derived from a healthy control

Quantitative real-time PCR (RT-PCR) assay for gene expression of *OCT4, KLF4, MYC, SOX2, NANOG, REX1, GDF3,* and *hTERT* was performed in iPSCs derived from healthy contriol and from patients with PID, and compared to the pattern observed in a previously established control human iPSC line, obtained by reprogramming using 4 different retroviral vectors[17]. Quantitative RT-PCR reactions were normalized against β-actin *(ACTB).* Expression was calculated using the ddCT method relative to expression levels in the individual parent fibroblast cell lines for the normal and PID-specific iPSC or normal control human fibroblasts for the human ES cells.

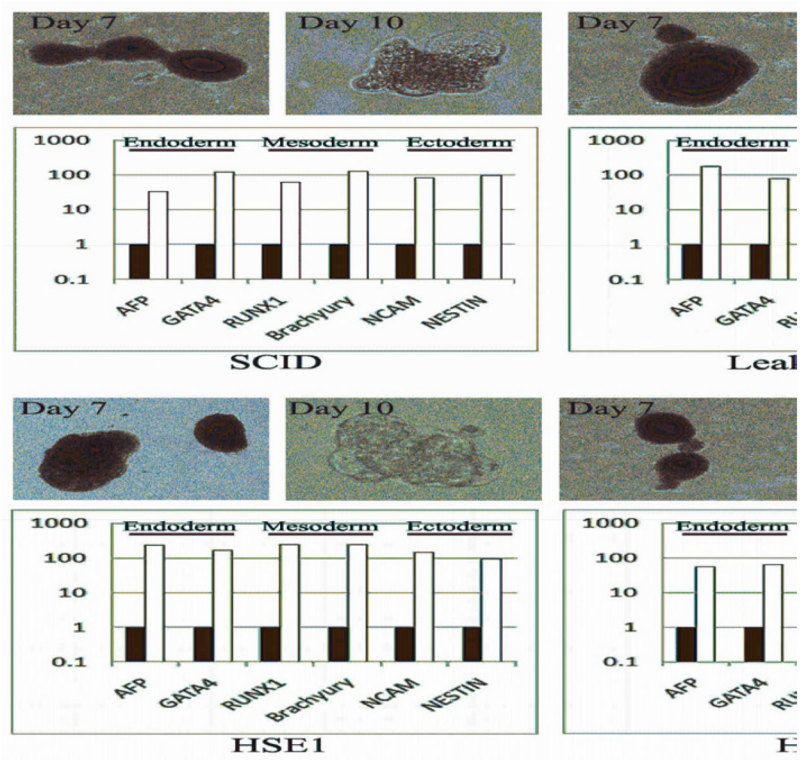

Figure 3. Differentiation of PID-specific iPSCs reveals lineage-specific gene expression

PID-specific iPSC were allowed to differentiate into embryoid bodies (EB) by culture in a bFGF-free hES medium and without co-culture with feeder cells. Robust formation of tight and well-formed cell clusters was detected by day 7, that became cystic by day 10 (upper row in each cell-specific panel). Quantitative RT-PCR gene expression analysis of the derived EB after 10 days shows increased expression of lineage-specific markers from each of the three embryonic germ layers, including: *AFP* and *GATA4* (endoderm), *RUNX1* and *Brachyury* (mesoderm), *NCAM* and *NESTIN* (ectoderm). Quantitative RT-PCR reactions were normalized against β-actin *(ACTB).* Expression was calculated using the ddCT method relative to expression levels in undifferentiated iPSCs. Black and white bars identify undifferentiated iPSCs and day 10 EB, respectively.

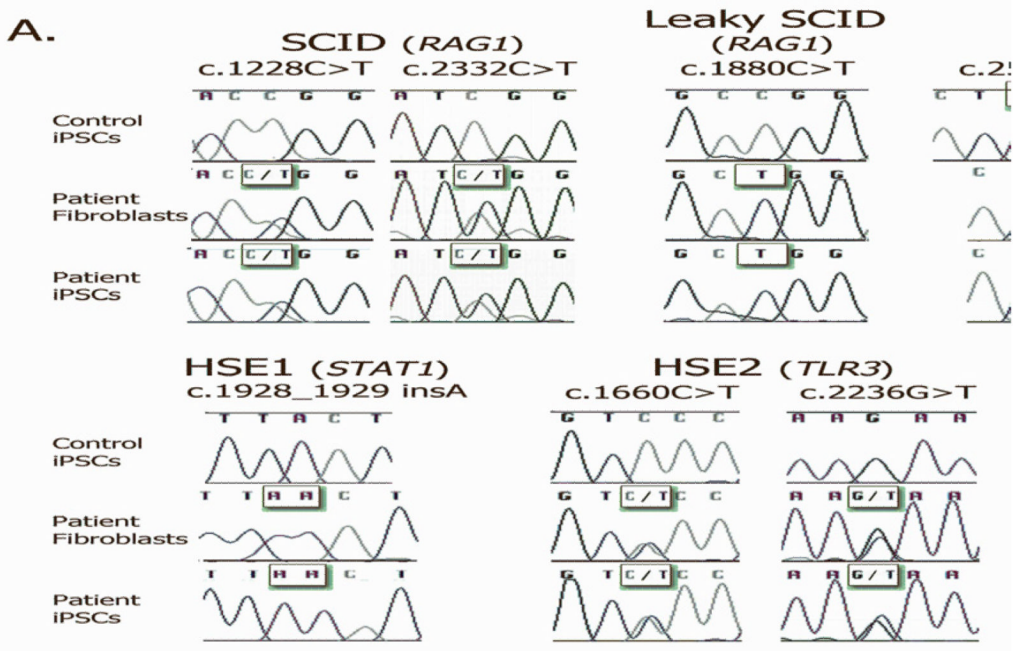

Figure 4. Patient origin and chromosomal integrity of the PID-specific iPSCs

A) PCR amplification followed by DNA sequencing of genomic DNA derived from the PID-specific iPSCs and their parental fibroblasts was performed using specific primers corresponding to the disease-causing mutations for each of the lines, and demonstrated that the PID-specific iPSC lines carry the same disease-causing mutations as their parental fibroblasts. In the case of the first allele (c.256-257del) of the *RAG1*-mutated patient with Omenn syndrome, genetic identity between patient-derived iPSCs and fibroblasts was demonstrated upon cloning and sequencing of the specific product.

B) PID-specific iPSCs were analyzed for chromosomal integrity by G-banding karyotyping.

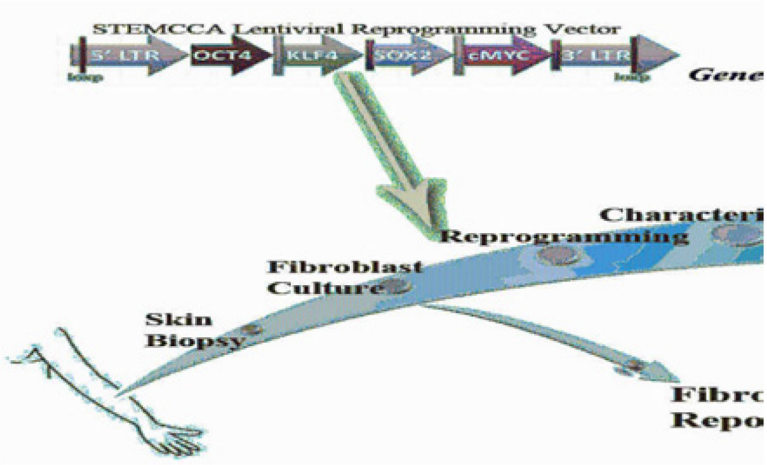

Figure 5.
Schematic representation of the process of producing PID-specific iPSCs and their possible use in disease characterization and gene correction.

Table I

Patients and mutations

Disease Phenotype	Gene	Mutation	Reference
SCID	*RAG1*	c.1228C>T; c.2332C>T	unpublished
Leaky SCID	*RAG1*	c.1180C>T; c.1180C>T	unpublished
OS	*RAG1*	c.256-257del; c.2164G>A	59
HSE1	*STAT1*	c.1928_1929 insA; c.1928_1929 insA	60
HSE2	*TLR3*	c.1660C>T; c.2236G>T	Guo Y *et al.*, submitted
CHH	*RMRP*	c. 27G>A; c. 27G>A	unpublished

SCID: Severe combined immunodeficiency; OS: Omenn syndrome; HSE: Herpes Simplex Encephalitis; CHH: Cartilage Hair Hypoplasia; *RAG1*: Recombinase Activating Gene 1; *STAT1*: Signal Transducer and Activator of Transcription 1; *TLR3*: Toll-like Recepetor 3; *RMRP*: RNA component of the Mitochondrial RNA Processing endoribunuclease