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Patient-specific Induced Pluripotent Stem Cells as a Platform for Disease Modeling, Drug Discovery and Precision Personalized Medicine

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Abstract

The breakthrough development of induced pluripotent stem cell (iPSC) technology is not only revolutionizing basic stem cell science but is also spurring efforts to reprogram one somatic cell type directly into another. Induced pluripotent stem cells provide scientists with a self-renewing and, thus, unlimited, source of pluripotent cells for targeted differentiation, in principle, into the entire range of cell types found in the body. Therefore, iPSC technology and the increasingly refined abilities to differentiate iPSCs into disease-relevant mature cells has far reaching implications for understanding disease etiology and promoting drug discovery and other advances in regenerative medicine. In this review, we summarize the latest progress in the application of patient-specific iPSCs for disease modeling, drug screening and cell replacement therapy, and discuss their impact on precision personalized medicine.

Keywords: Induced pluripotent stem cell; Reprogramming; Patient-specific; Disease modeling; Drug discovery; Therapeutics; Precision personalized medicine

Introduction

The establishment of human embryonic stem cells (hESCs) in 1998 together with their unlimited self-renewal potential and ability to differentiate into any cell type of the body has raised hopes for drug discovery and regenerative medicine [1]. However, the challenges related to bioethics, safety and the limited availability of disease-specific hESC lines have complicated the realization of these hopes. This changed dramatically in 2006 when Kazutoshi Takahashi and Shinya Yamanaka made the seminal discovery that mouse skin fibroblasts can be reprogrammed into an induced pluripotent stem cell (iPSC) state that shares the indefinite self-renewal and pluripotent differentiation capacities of ESCs using a simple cocktail of pluripotency transcription factors [2]. One year later, these same investigators as well as groups headed by James Thomson and George Daley succeeded in converting human fibroblasts into hiPSCs [3-5]. Reprogramming to pluripotency has now been demonstrated starting with a variety of somatic cell types [6-13]. Taken together these advances enable the generation of patient- and disease-specific hiPSCs as avenues for exploring disease etiology, developing novel drugs, toxicology screening and, in the future, cell replacement therapies.

Reprogramming Cell Fates

Historically the ground-work for reprogramming was established by Hans Spemann in 1938 [14]. Using a hair to constrain the nucleus to one side of a newly fertilized salamander egg he was able to show that the side with the nucleus could divide but not the side without it. Once the hair was loosened, after four cell divisions creating a 16-cell embryo, the nucleus slipped back into the separated cytoplasm, and cell division began on this side as well. After a few divisions, Spemann then tightened the hair loop again, and broke apart the two embryos. A twin set of salamanders developed, one slightly younger than the other. This experiment demonstrated that the nucleus retains totipotency even after having undergone four divisions. It was not until 1952 that Spemann's "fantastical experiment", the transfer of a nucleus from an older embryo into an egg, was successfully executed by Briggs and King [15]. The two authors described the development of normal embryos after the transplantation of nuclei from advanced blastula cells into enucleated eggs of the frog *Rana pipiens*. This was truly the first successful nuclear transfer experiment. Ten years later Sir John Gurdon using the *Xenopus* system transferred terminally differentiated intestinal epithelial cell nuclei into unfertilized eggs and demonstrated that 1.5% of the transferred nuclei successfully developed into tadpoles [16]. This experiment demonstrated that nuclei from terminally differentiated cells remain totipotent. Sir Ian Wilmut reported the successful creation of a cloned mammal in 1997 when he and his colleagues cloned Dolly the sheep by fusing a somatic donor cell nucleus with an enucleated oocyte [17]. In 1998, Wakayama and colleagues reported similar results in mice by injecting cumulus cell nuclei into enucleated metaphase II oocytes [18].

Nuclear Reprogramming, the process that reverts the epigenetic state of a nucleus particular to one specialized cell type to that of a different cell type, can be achieved by heterokaryon formation. Helen Blau and colleagues established the stable heterokaryon system by fusing human amniotic, non-muscle cells with terminally differentiated multinuclear mouse myotubes in 1983 [19]. These heterokaryons did not divide and retained two complete sets of chromosomes. Nevertheless, the mouse muscle cell nuclei were sufficient to activate human muscle-specific genes. Acquiring pluripotency in a somatic cell was first described by M. Azim Surani and colleagues by fusing mouse thymic lymphocytes with embryonic germ cells [20]. The hybrid cells showed dramatic

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changes in the epigenetic state of the somatic cell nucleus that now resembled the germ cell state. In addition, the hybrid cells were able to differentiate into a variety of tissues in chimeric embryos thus proving functional pluripotency. In 2001, Takashi Tada's group demonstrated that ESCs contain the factors required to reprogram somatic cell nuclei by fusing thymocytes with ESCs [21].

Reprogramming by ectopic expression of "master regulator" genes was first described in 1987 by Walter Gehring's group in *Drosophila* [22]. Flies, with ectopically expressed homeotic *Antennapedia* gene, developed secondary legs instead of antennae. In the same year, Harold Weintraub and colleagues demonstrated that ectopic expression of a single myogenic basic helix-loop-helix transcription factor MyoD in non-muscle cells induced myogenesis, and stably converted C3H10T1/2 fibroblasts into myoblasts [23].

Reprogramming of Somatic Cells to Induced Pluripotent Stem Cells

In 2006, Yamanaka and Takahashi hypothesized that somatic cells could revert into a pluripotent, self-renewing ESC state when exposed to a defined set of factors expressed in ESCs and cultured in conditions that favor pluripotent cell growth. Using a retroviral system, they forced expression of a selected set of 24 candidate genes in mouse embryonic fibroblasts (MEFs) and were successful in establishing clones that possessed ESC-like morphologies, proliferation rates, expressed ESC markers and had demethylated the promoter of the *Nanog* gene [2]. These cells were termed as induced pluripotent stem cells (iPSCs). Removing one factor at a time Takahashi and Yamanaka further demonstrated that a minimum set of only four factors namely, Klf4, cMyc, Oct4 and Sox2 were necessary for reprogramming MEFs as well as tail-tip fibroblasts from adult mice into iPSCs. Later studies showed that the presence of cMyc is not an absolute reprogramming requirement but its absence significantly reduces the efficiency of the process [24].

The successful reprogramming of human somatic cells to hiPSCs was reported within one year [3-5]. Takahashi and Yamanaka, as well as Daley's group used KLF4, cMYC, OCT4 and SOX2, the same factors as in the mouse system, to convert human fibroblasts into iPSCs [3,4]. The Thomson group achieved the same results using LIN28, NANOG, OCT4 and SOX2 [5].

Since then the field of cellular reprogramming has progressed at an unprecedented pace. Specific advances include the use of various cell types, including, keratinocytes, terminally differentiated neurons, lymphocytes, hair follicle dermal papilla cells and even tumor cells for reprogramming [6-13], and the replacement of individual factors by other regulators [25-29], small molecules [27,30-40] or a modified culture condition [41]. There are also studies that provide insights into the molecular mechanism of the reprogramming process [30,42-48]. To facilitate the transition of this technology into the clinic, the vector system has gradually evolved from integrating viral systems, which may cause insertional mutagenesis and thus pose a risk for translational application of the technology, to non-integrating systems as evidenced by the use of adenovirus [49], plasmids [50-52], excisable viruses [53-55], episomal or minicircle vectors [56,57], piggyBac transposons [58-60], proteins [61], Sendai virus [62], synthetic mRNAs [63], and microRNA mimics [64].

Furthermore, iPSC lines have been derived from species other than mice or humans, including rhesus monkey [65], marmoset [66] rat [34,67], pig [68-70], dog [71,72], sheep [73], horse [74] and cow [75]. These lines will allow us to genetically engineer these animals, facilitating the generation of large(r) animal disease models. An additional potential use of iPSC technology is the preservation of endangered species [76], possibly extending to the resurrection of extinct ones. Of course a number of obstacles, including suitable embryo culture and identification of an appropriate surrogate mother species need to be resolved before this can become a reality.

Direct Reprogramming of One Somatic Cell Lineage to Another

As mentioned above, successful reprogramming of mammalian somatic cells to myoblasts by ectopic expression MyoD was already reported in 1987 [23]. Thomas Graf and his group succeeded in identifying the minimal set of transcription factors sufficient to trans-differentiate one hematopoietic cell lineage into another. Gata1 was sufficient to convert myelomonocytic cells into erythrocytes [77], while Cebpa or Cebpb transformed B-lymphocytes into macrophages [78]. Transduction of both Cebpa and Sfpi1 (PU.1) was required to switch T cells into macrophages [79].

It took the success of iPSC reprogramming to rekindle a broad interest in direct reprogramming of one somatic cell lineage into another. Because attempts to identify a single "master" regulator have failed for most cell lineages, scientists started to look for combinations of factors. The first such successful reprogramming was surprisingly achieved *in vivo* by Doug Melton and his group, by converting differentiated pancreatic exocrine cells in adult mice into cells that closely resembled endocrine β-cells with a combination of three transcription factors [80]. This seminal study was soon followed by a flurry of *in vitro* examples of reprogramming fibroblasts into various distinct somatic cell lineages, including, neurons [81-85], neural progenitors [86], neurosphere-like cells [87], neural stem cells [88-90], hepatocyte-like cells [91,92], and cardiomyocytes [93-97]. In addition to the conversion of pancreatic exocrine to endocrine cells [80], *in vivo* somatic cell reprogramming has so far been achieved only for cardiomyocytes [94,97,98]. Direct reprogramming is straightforward and rapid. One remaining obstacle is low efficiency precluding the generation of sufficient target cell numbers for downstream applications (see below).

Sheng Deng's group demonstrated an alternative approach for directed lineage conversion from fibroblasts to another somatic cell type by transient expression of the four Yamanaka factors followed by culture conditions favoring non-pluripotent, specialized cell types such as cardiomyocytes and neural progenitors [99,100]. In this case, it is possible that the factors serve to erase the epigenetic fibroblast state and the culture conditions select for random or stochastic epigenetic resetting events. This is also the likely mechanism for the reported generation of hematopoietic progenitors after expression of Oct4 [101].

Disease Modeling

Conventional human disease research is performed using platforms such as epidemiology, genetics, genomic and epigenomic profiling, animal models as well as various, sometimes heterologous, *in vitro* cell culture models. However, these approaches are often accompanied by several complications. First, human tissue or cell samples are often difficult to obtain, sometimes requiring invasive surgery as in the case of the heart or brain, or available only after the patient's death. Second, generally the isolated cells cannot be maintained or expanded with current culture conditions, except if rendered immortal. However, immortalization by genetic manipulation alters normal cell physiology and thus renders any data and analyses obtained from the modified

cells, highly questionable. Third, animal models are only suitable if the physiology of the experimental species is comparable to humans. And fourth, heterologous cell culture models, although convenient, easily accessible, and widely utilized will nevertheless lack the typical biological, physical and physiological features of the diseased human cell. The technology of patient- or disease-specific iPSC derivation coupled with directed differentiation to appropriate target cells circumvents these obstacles and provides several additional advantages. First, hiPSCs can be generated from patients with genetic diseases and, therefore, the derived target cells thus possess the same genetic background as the patient. This is important because an individual's genetic makeup can profoundly influence disease progression, its severity, as well as the elicited drug response. One example of an adverse effect of the latter is drug-induced cardiac arrhythmia. Second, the ability of correct genetic disease-associated lesions (see below) in patient-derived iPSCs increases their potential for eventual cell-based therapies. In a proof-of-principle study Hanna and colleagues demonstrated that a mouse sickle cell anemia model could be treated by transplantation of hematopoietic progenitor cells derived from "autologous" iPSCs with a repaired sickle hemoglobin allele [102].

Disease Selection, Patient Recruitment and Cell Sampling

The first step in hiPSC-based disease modeling is choosing a disease or syndrome. Careful selection of a disorder for patient-specific iPSCs disease modeling is a critical step and may predetermine the likelihood of success. In general, genetic disorders are easier to model than those caused by predominant epigenetic or environmental factors. Monogenetic disorders with a clear disease phenotype are easier to model than complex genetic disorders. The same holds true, for congenic or familial diseases compared to sporadic diseases. Nevertheless, there are clear examples of successful hiPSC-based disease modeling of complex disorders, including sporadic Alzheimer's disease [103] and Schizophrenia [104,105] (see also Table 1). After choosing a disease the next step is applying for internal review board approval to recruit patients and conduct the study. With approval, suitable patients are recruited, informed about the process and study, their consent obtained, and finally skin, blood or other tissue/cell samples are collected. The least invasive way to obtain cells is by drawing blood from the patient. Another convenient and easy way is to perform a skin biopsy under local anesthesia using a 2-4mm biopsy punch without a suture. Once the tissue or cell samples are collected, they need to be expanded. For example, one method involves the separation of the buffy coat fraction from blood, followed by expansion of T-cells in the presence of IL-2 and activating antibodies CD3 and CD28 for 4-6 days [12]. One fraction of these cells are used for quality control assays, another fraction banked for future DNA finger printing studies and one fraction used for iPSC generation. Fibroblasts, on the other hand, can routinely be grown from skin biopsies, expanded using standard tissue culture conditions, and then subjected to similar quality control and banking procedures as described for blood cells.

Patient-specific iPSC Derivation, Expansion and Banking

Reprogramming can be achieved using the various methods described above. For simplicity and reproducibility, we prefer the non-integrating Sendai virus or Stemgent's latest synthetic mRNA/microRNA transfection methods (Brad Hamilton, Stemgent, presentation at ISSCR 10th Annual Meeting, June 13-16, 2012). Both methods are similarly robust for reprogramming patient- or disease-specific fibroblasts. Reprogramming of blood-derived cells is currently only feasible using Sendai virus but this technical issue will likely be resolved in the near future. Reprogramming with mRNA/microRNA has the advantage that once hiPSCs are established they can be used immediately for downstream studies. In contrast, hiPSCs derived using Sendai virus need to be selected for absence of the virus, which can take up to 8-10 passages or 6-10 weeks. If patient-specific iPSCs or their derivatives are considered for cell replacement then the derivation process needs to be performed under Good Manufacturing Practice (GMP) conditions.

Irrespective of the derivation method, properly reprogrammed clones need to be identified and expanded. This requires familiarity with pluripotent stem cell (PSC) morphology and proper passaging techniques. An inherent problem of hiPSC derivation and expansion is that individual hiPSC lines are heterogeneous with respect to their growth properties and differentiation abilities. The reasons for such heterogeneity are currently unclear. Selection of accurately reprogrammed, "true" hiPSC lines may be facilitated by "live"-staining for pluripotency-associated cell surface markers such as TRA-1-60 or TRA-1-81 [106]. Established hiPSC lines need to be DNA fingerprinted to ensure that they originate from the donor cell source and are not contaminated by other cell lines. In addition, the newly generated hiPSC lines need to fulfill a set of criteria to confirm complete reprogramming and pluripotency [107-109]. These include PSC morphology, unlimited self-renewal, expression of molecular pluripotency-associated markers, pluripotency gene promoter demethylation, and functional *in vitro* and/or i*n vivo* ability to differentiate into derivatives of to all three germ layers. Currently there is a need for a consensus minimal set of criteria that can be used in multiple studies. This will enable rigorous cross-laboratory comparisons. Furthermore, such a minimal set of criteria is necessary for studies focused on complex, multi-factorial diseases that will require the recruitment of many patients. As an example, currently many groups perform *in vivo* teratoma assays in immunodeficient mice to assess pluripotency. This is costly and labor intensive and clearly, cannot be routinely performed for numerous hiPSC lines. This as well as other criteria are currently the subject of lively discussions [107,108]. There is also an ongoing debate as to how many passages are required to completely erase the epigenetic programs of the starting cell populations [110,111]. In addition, selection of high-quality patient-specific iPSC lines would be dramatically improved by identification of molecular markers that would predict hiPSC differentiation potential.

Established hiPSC lines that meet to the above criteria need to be expanded for banking. Before cryopreservation, expanded lines should be tested for a normal karyotype. However, standard karyotyping methods will not detect small chromosomal aberrations or point mutations, and more sophisticated approaches such as comparative genomic hybridization or whole-genome sequencing may be required. Currently, these are costly but are rapidly becoming affordable. Overall, the extent of necessary characterization of iPSC lines may also be guided by specific downstream applications.

Disease Correction

Genetic correction of the disease-associated mutation(s) in patient-specific iPSC lines serves several purposes. First, it will generate isogenic control cells that differ only in the particular mutation(s) from the original patient-specific iPSC line. These disease-corrected hiPSCs are the perfect control for any comparative analyses of disease phenotype. Second, these disease-corrected hiPSCs and their differentiated target cells could eventually be applied in cell replacement therapies.

Disorder	Affected Gene(s)	Phenotype assessment	Reference(s)
α1-antitrypsin deficiency	*SERPINA1*	Aggregation of misfolded alpha1-antitrypsin in the endoplasmic reticulum	[182]
Adrenoleukodystrophy, X-linked	Unknown	iPSC-oligodendrocytes exhibit very long chain fatty acid level	[183]
Alzheimer's disease, familial	*PSEN1*, *PSEN2* *APP*	iPSC-neurons have increased amyloid β42 secretion, phosphorylated-Tau and active glycogen synthase kinase-3β as well as accumulation of large $Rab5^+$ early endosomes	[103,154]
Alzheimer's disease, sporadic	Unknown	Increased levels of amyloid-β, phosphorylated-Tau and active glycogen synthase kinase-3β as well as accumulation of large $Rab5^+$ early endosomes	[103]
Amyotrophic lateral sclerosis, familial	*TARDBP* or *VAPB*	TARDBP iPSC neurons have elevated levels of soluble and detergent-resistant TDP-43 protein, decreased survival in longitudinal studies, and increased vulnerability to antagonism of the PI3K pathway; VAPB fibroblasts, iPSCs and hiPSC neurons have reduced levels of VAPB	[184,185]
Catecholaminergic polymorphic ventricular tachycardia type 1	CASQ2 or *RYR2*	iPSC cardiomyocytes are arrhythmogenic, have delayed afterdepolarizations, after-contractions, and exhibit higher amplitudes and longer durations of spontaneous Ca^{2+}-release	[150,186-188]
Chronic granulomatous disease, X-linked	*CYBB* or *NCF1*	iPSC neutrophils or iPSC-macrophages lack ROS production	[189,190]
Chronic infantile neurological cutaneous and articular syndrome	*NLRP3*	iPSC marcophages show abnormal IL1β secretion	[191]
Chronic myelogenous leukemia	*BCR-ABL*	iPSC mature but not immature hematopoietic cells are sensitive to imatinib	[192]
Dilated cardiomyopathy	*LMNA or Tnnt2*	iPSC-fibroblasts have nuclear membrane abnormalities, increased senescence and susceptibility to apoptosis; iPSC-cardiomyocytes show altered regulation of Ca^{2+}, decreased contractility, abnormal distribution of sarcomeric α-ACTININ, and cellular stress upon stimulation with a β-adrenergic agonist	[193,194]
Down syndrome	Trisomy 21	iPSC teratoma microvessel density is significantly reduced (reference 195); iPSC-neurons processed the transmembrane APP protein, resulting in secretion of the pathogenic peptide fragment amyloid-β42, a feature of Alzheimer's disease (reference 196)	[195,196]
Dyskeratosis congenita	*DKC1, TERC* or *TCAB1*	iPSC show progressive telomere shortening and loss of self-renewal	[197,198]
Familial Dysautonomia	*IKBKAP*	Decreased expression of genes involved in neurogenesis and neuronal differentiation; defects in neural crest migration	[151]
Familial Hypercholesterolemia	*LDLR*	hiPSC-derived hepatocytes have an impaired ability to incorporate LDL, increased secretion of lipidated ApoB-100	[182,199]
Fragile X syndrome	*FMR1*	iPSC show aberrant neuronal differentiation with loss of expression of *FMRP*	[200]
Gaucher's disease type III	*GBA*	iPSC-neurons have compromised lysosomal protein degradation, accumulation of α-SYNUCLEIN and neurotoxicity through aggregation-dependent mechanisms	[201]
Glycogen storage disease type 1A	*G6PC*	iPSC-hepatocytes hyperaccumulate glycogen and lipid, and have excessive production of lactic acid	[182]
Huntington's disease	*HTT*	iPSC-neural stem/progenitor cells show enhanced caspase activity upon growth factor deprivation; iPSC asctrocytes show cytoplasmic vacuolation; iPSC but not iPSC neurons have significantly increased lysosomal activity	[115,202-205]
Hurler syndrome (Mucopolysaccharidosis type I)	*IDUA*	iPSC have an imbalance between production and clearance of unprocessed GAG and show lysosomal storage defects	[206]
Hutchinson-Gilford progeria	*LMNA*	iPSC mesenchymal stem cells, vascular smooth muscle cells and fibroblasts display progerin accumulation, increased DNA damage, and nuclear abnormalities; iPSC-vascular smooth muscle cells also show premature senescence, blebbing and increased apoptosis	[193,207,208]
Juvenile Diabetes *	*LDLR*	iPSC hepatocytes show features of hypercholesteremia	[199]
LEOPARD syndrome	*PTPN11*	iPSC cardiomyocytes reveal features of cardiac hypertrophy (increased size, increased sarcomere organization and increased nuclear localization of NFATC4)	[140]
Long QT 3 and Brugada overlap syndrome	$SCN5A^{1798insD/+}$	iPSC cardiomyocytes show reduced upstroke velocity and longer action potential duration	[209]
Long QT syndromes	*KCNQ1* (LQT1); *KCNH2* (LQT2), *SCN5A* (LQT3), *CACNA1C* (LQT8/ Timothy syndrome)	iPSC cardiomyocytes have a prolonged action potential duration reminiscent of delayed repolarization, early afterdepolarization (LQT3), and irregular contraction, excess Ca^{2+} influx, irregular electrical activity and abnormal calcium transients (LQT8/ Timothy syndrome)	[210-215]
Machado-Joseph disease	*ATXN3*	iPSC neurons show formation of SDS-insoluble aggregates after Ca^{2+}-dependent proteolysis of ATXN3	[216]
Marfan syndrome	*FBN1*	iPSC show inhibition of osteogenic differentiation, enhanced TGFβ-signaling, and chondrogenic differentiation without TGFβ1 media supplementation	[217]

Mucopolysaccharidosis type IIIB	*NAGLU*	iPSCs and differentiated neurons derived from patients show defects in storage vesicles and Golgi apparatus	[218]
Parkinson's disease, familial	*LRRK2*, *SNCA* or *PINK1*	iPSC dopaminergic neurons show impaired mitochondrial function; iPSC dopaminergic neurons have increased expression of key oxidative stress response genes and α-synuclein protein, and increased sensitivity to oxidative-stress; accumulation of autophagic vacuoles	[156,219-223]
Polycythaemia vera	*JAK2*	iPSC hematopoietic cells have enhanced erythropoiesis	[224]
Pompe disease	*GAA*	iPSC cardiomyocytes show high levels of glycogen and multiple ultrastructural aberrances	[225]
Prader-Willi syndrome	Translocation of the paternally expressed chromosome 15q11-q13 region to chromosome 4	iPSCs express reduced levels of the disease-associated small nucleolar RNA HBII-85/ SNORD116	[226]
Retinitis pigmentosa	*RP1*, *RP9*, *PRPh2* or *RHO*	iPSC rod cells numbers are decreased, express markers for oxidation or endoplasmic reticulum stress, and show different responses to vitamin E	[227]
Rett Syndrome	*MECP2*	iPSC neurons have decreased synapse number, reduced number of spines, a reduction in soma size, altered calcium signaling, and elevated LINE1 retrotransposon mobility	[153,228-231]
Schizophrenia	Unknown	iPSC neurons showed diminished neuronal connectivity in conjunction with decreased neurite number, PSD95-protein levels and glutamate receptor expression; iPSC neurons have an increase in extra-mitochondrial oxygen consumption as well as elevated levels of reactive oxygen species	[104,105]
Sickle cell disease	*HBB*	Marginally increased HBB mRNA levels	[117]
Spinal muscular atrophy	*SMN1*	iPSC motor neurons have a reduced size and decrease in numbers over time; show an abnormality in neurite outgrowth	[152,232]
Timothy syndrome	*CACAN1C*	iPSC cardiomyocytes have irregular contraction, excess Ca^{2+} influx, prolonged action potentials, irregular electrical activity and abnormal calcium transients iPSC cortical neurons have defects in Ca^{2+}-signaling and activity-dependent gene expression, as well as abnormalities in cortical neuron differentiation	[212,233]
Werner syndrome, atypical	*LMNA*	iPSC fibroblasts show nuclear membrane abnormalities, increased senescence and susceptibility to apoptosis	[193]
Wilson's disease	*ATP7B*	iPSC hepatocytes mislocalize mutated ATP7B and have defective copper transport	[234]

*see also Familial Hypercholesteremia

Table 1: Patient-specific iPSCs with described disease phenotypes.

There exist several molecular approaches for gene targeting to correct and also introduce genetic mutations into the genome of cells. The classical approach is using homologous recombination technology. This method is successfully used in mouse PSCs but is very inefficient in hPSCs as demonstrated by the small number of published reports (see references in [112]). Nevertheless, three recent reports have demonstrated the feasibility of using classical homologous recombination to repair an underlying genetic mutation in patient-specific iPSCs. In one case for gyrate atrophy [113], in another case for β-thalassemia major or Cooley's anemia [114], and in the third case for Huntington's disease [115]. A second approach to genetically correct a disease-associated mutation in hiPSCs is by using the highly efficient system of helper-dependent adenovirus-mediated homologous recombination [116]. A third way of gene-editing can be accomplished using site-specific zinc finger nucleases (ZFNs) in combination with an exogenous donor vector that carries homologous sequences needed for homology-directed repair of the ZFN-mediated precise DNA double strand break. Incorporation of a drug resistance cassette into the donor vectors allows for selection of targeted cells and thus increases the success rate. Flanking of this cassette with either loxP or FRT sites allows for its removal by transient expression of Cre- or Flp-recombinase, respectively. This approach has been successfully employed in repairing disease-associated mutations in patient-specific iPSCs, namely for the mutated *β-GLOBIN* allele causing sickle cell disease [117,118] or the dominant A53T mutation in *α-SYNUCLEIN* that is associated with Parkinson's disease [119]. An alternative, similar to ZFN in approach and robustness, but apparently simpler technique is transcription activator-like effector nuclease (TALEN)-mediated gene targeting. TALENs are designed after natural TALEs, a class of DNA binding proteins expressed by plant pathogenic bacteria that can subvert the host's genome regulatory network [120]. TALENs that recognize precisely predetermined sequences in the genome can be easily designed and assembled. They have been successfully used to efficiently modify the genomes of many species and cell types, including hESCs and hiPSCs [121]. However, to date no report exists of TALEN-mediated gene correction in patient-specific hiPSC. This is clearly only a matter of time.

RNA interference (RNAi) technology can be an innovative approach to rescue disease-associated phenotype(s). Because of its potency and high mRNA target specificity, RNAi provides an ideal opportunity to inhibit alleles of genes showing inherited or acquired polymorphisms and alternative or cryptic splicing with single point mutations found in inherited diseases. The functional utility of RNAi-mediated selective suppression of mutant mRNAs in dominant human genetic disorders has been established (for examples see references in [122]). The feasibility of RNAi to revert a disease-phenotype in patient-specific iPSCs has been demonstrated in the neurological disorder Friedreich's ataxia where shRNA silencing of the mismatch repair enzyme MSH2 impeded the disease-associated GAA/TTC repeat expansion [123].

Directed Differentiation

Disease modeling, drug discovery and future cell replacement therapies rely heavily on efficient and homogeneous differentiation of hiPSCs to appropriate specialized cells. Many years of work have defined protocols for differentiation of hESCs and more recently hiPSCs to distinct cell types, including neurons, cardiomyocytes and hepatocytes, mimicking pathways of human development. Protocols for differentiation to more specialized cell subsets, such as nodal, atrial, ventricular or Purkinje fiber cardiomyocytes, have not yet been established but will be critical for modeling specific disorders and for drug discovery. In many cases the differentiation process has been developed for particular cell lines and often is not similarly robust in a broad range of hPSC lines without individual, line-specific modifications. In addition, the process is often inefficient and produces a heterogeneous cell population consisting of multiple cell types and/or a mixture of cells at different developmental stages, mostly consisting of fetal or immature phenotypes (to name a few examples: cardiomyocytes [124,125], dendritic cells [126], neural cells [127], and pancreatic β-cells [128]). Small molecules that mimic growth factors and directly activate developmental programs would reduce not only the cost of differentiation (recombinant growth factors are expensive) but also alleviate the batch-to-batch variability in protein activity. Unfortunately, small molecules do not yet exist for most growth and developmental conditions. Nevertheless, the number of reports describing differentiation protocols that incorporate or exclusively use small molecules is growing [129-133] and development of such protocols is part of ongoing research in numerous laboratories. Another complicating component that contributes to variability in disease modeling or drug discovery is the multistep nature of the differentiation process that can take several weeks or even months depending on the specific target cell population. Overall, the single most critical consideration for any large(r)-scale analysis or screening purpose is the ability to scale-up the expansion and differentiation process and retrieve pure specialized target cells. Demonstrating the feasibility of the former has primarily been concentrated on the reproducible and homogeneous proliferation of undifferentiated hPSCs using bioreactors [134-137], but there are only a few reports on scalable, controlled and regulated differentiation [134,138,139]. Pure differentiated cell populations can be achieved by enrichment using fluorescence-activated cell sorting or magnetic bead separation. However, for many specialized human cells appropriate surface antigens and corresponding antibodies have not yet been identified. In addition, certain target cells may require co-culture with support/niche cells for proper differentiation, maturation or survival.

Disease Modeling: Identification and Analysis of Cellular Disease Phenotypes

The concept of *in vitro* disease modeling with patient-specific iPSCs is very attractive and is based on their unique ability to self-renew indefinitely, their potential to give rise to all cell types in the body and their matching genetic identity with a particular patient. However, the key to any disease modeling is the availability of a measurable disease-relevant phenotype, such as electrophysiological abnormalities in cardiomyocytes derived from cardiac disease patient-specific iPSCs. Measurable phenotypes are more likely to be found in monogenetic disorders with a clearly identified disease phenotype than in complex genetic disorders such as Autism, which are more complicated to model. Furthermore, to control for the inherent variability among hiPSC lines derived from the same patient several lines (generally, at least three) should be examined. A pressing and re-occurring issue in the field of hiPSC-based disease modeling is "What constitutes an appropriate control cell?" relative to which the obtained analytical data can be evaluated. Human iPSC lines derived from un-affected close relatives, i.e. parents, siblings or children, that share 50% identity of the genetic information with the patient, are considered suitable controls. However, at least for monogenic Mendelian disorders, patient-specific iPSCs in which the underlying disease-associated mutation has been corrected (see above) are undoubtedly the best controls because of their isogenic nature, aside, of course, from the mutation. Despite the concerns raised above, successful modeling of several monogenic, familial and even sporadic disorders with patient-specific iPSCs has been accomplished (Table 1). We were part of the team that established the first human cardiac disease model with LEOPARD syndrome (LS) patient-specific iPSC by modeling the hypertrophic cardiomyopathy phenotype found in LS patients with mutations in the *PTPN11* gene [140].

Future research will undoubtedly move towards the comparison of hiPSC lines from many patients with the same disorder and/or mutation. Such comparative analyses will help address whether inter-patient differences in clinical disease progression and severity can be modeled *in vitro*. If this were the case, and we are confident that this is possible, then comparative disease modeling opens the door to the search for the underlying genetic and/or epigenetic component(s) responsible for these differences. Along the same line, hiPSCs derived from select groups of people with acquired symptoms/disorders such as drug-induced arrhythmias or insulin-resistance, and an appropriately selected non-affected control population may enable the identification of the respective underlying mechanism(s). Clearly, there is great potential here for combining hiPSC disease modeling with genome-wide association studies (GWAS) to uncover assemblies of loci that collectively contribute to complex multifactorial diseases [141-143]. Given the complexity of such diseases, issues such as the ability to generate numerous hiPSC lines from many patients, the development of robust differentiation protocols, minimizing line to line variability, obtaining suitable numbers of controls from unaffected individuals, etc. become extremely relevant. With large numbers of hiPSC lines from affected and unaffected populations coupled with the power of GWAS it may be possible to identify causative or predisposing genetic loci using powerful statistical approaches [144,145]. Understanding these genetically- and/or epigenetically-influenced disparities between individual groups may lead to better diagnostic predictors, possibly, earlier detection and hopefully the development of fine-tuned, personalized therapeutics.

Drug Evaluation, Discovery and Development

Currently, the development of pharmaceutical therapeutics is a very inefficient process. A large majority of identified candidate drugs fail to reach the market because of safety concerns (about one third of pharmaceuticals are withdrawn due to cardiotoxicity [146]) and efficacy issues. The weakness in the current drug development model is that drug responses measured in animal models cannot be used to predict efficacy in humans. Genetically modified rodents and immortalized human cell lines also fail to replicate human conditions. In contrast to these systems, iPSC technology can accurately replicate disease-specific cell types within the disease-relevant genetic context allowing for better high-throughput screening assays to select for a candidate drug's therapeutic response as well as toxicity. Moreover, the use of hiPSC in screening could decrease the number of animals sacrificed during drug testing [147].

An early example of a hiPSC-based drug screen is from Tanaka and colleagues [148]. They differentiated hiPSC to cardiomyocytes expressing cardiac markers including NKX2-5, GATA-4 and atrial naturetic peptide as well as cardiac specific forms of sodium, potassium and calcium channels. Using multi-electrode arrays (MEAs), Tanaka and colleagues measured changes in the electrophysiological properties of these hiPSC-derived cardiomyocytes in response to different ion channel inhibitors namely, quinidine, verapamil, and a potassium channel blocker [148]. Their study showed that hiPSC-derived cardiomyocytes and native cardiomyocytes responded similarly to various pharmacologic agents. Another study showed the ability of hiPSC-derived cardiomyocytes to respond to amiodarone, isoproterenol, procainamide, mexilitine, and propranolol with respect to contractility and beating rate [149].

The pharmaceutical compound dantrolene was found to rescue the arrhythmogenic defect in a patient-specific iPSC model of catecholaminergic polymorphic ventricular tachycardia 1 (CVPT1) caused by the S406L mutation in the cardiac ryanodine receptor type 2 (*RYR2*) gene [150]. This receptor mutation alters the frequency and duration of elementary Ca^{2+} release from the sarcoplasmic reticulum upon catecholaminergic stress and, thus, causes elevated diastolic Ca^{2+} concentrations, a reduced sarcoplasmic reticulum Ca^{2+} content and an increased susceptibility to delayed after-depolarizations and arrhythmia when compared to control cardiomyocytes. The authors postulated correctly that dantrolene, which is believed to stabilize the interaction between the N-terminal and central domains of RYR2 could suppress the effect of the S406L mutation, which is positioned at the interface between these two regions. Treatment of S406L *RYR2* CVPT1 hiPSC-derived cardiomyocytes with dantrolene indeed restored normal Ca^{2+} sparks and fluxes.

Recently, the first high-throughput drug screening study was published employing hiPSC-derived cardiomyocytes [146]. Employing a new 96-well MEA, this was a novel method for quantifying drug-induced proarrhythmic risk. A monolayer of hiPSC-derived cardiomyocytes tested with 25 known pharmacologic agents evoked predicted responses from the cardiomyocytes. Furthermore, two known arrhythmogenic compounds induced arrhythmic impedance traces that were inhibited by calcium channel blockers. QT prolonging agents were also used and all of them induced a dose- and time-dependent irregular beating pattern reminiscent of torsade de pointes type arrhythmias. Therefore, at least in principle, iPSC derived cell lineages can be used not only for screening of therapeutic drug candidates but also for screening for the effects of known drugs.

Most studies employing hiPSCs in drug screening have been in neuronal diseases. Patient-specific iPSC lines have been created for various neuronal diseases such as familial dysautonomia (FD) [151], spinal muscular atrophy (SMA) [152], Rett syndrome [153], Alzheimer's disease [154,155] and Parkinson's disease [156] to name a few. Although the specific culture conditions needed for differentiation of hiPSCs into neuronal lineages for drug discovery applications are generally available, they require further refinements. Lee and colleagues modeled FD, a genetic disease of dysfunction of the autonomic and sensory nervous systems due to the incomplete development of and survival of sensory, sympathetic and some parasympathetic neurons caused by mutations in *IKBKAP* encoding for IkB kinase complex associated protein, using iPSC technology. Lee and colleagues found that the homozygous 2507+6C>T mutation in *IKBKAP* results in the expression of particularly low levels of normal, correctly spliced IKBKAP mRNA, defects in neurogenesis and neural crest cell migration [151]. Treatment of differentiating FD-iPSCs but not derived neural crest precursors with kinetin, a plant hormone, significantly increased the number of developing neurons and the expression of key peripheral neuron markers but not the neural crest cell migration defect. This study demonstrated that hiPSC-based drug discovery has good predictive value for therapeutics.

In a study focused on SMA, Ebert and colleagues employed patient-specific iPSC-derived motor neurons and astrocytes, recapitulating physiological characteristics of SMA with decreased expression of survival motor neuron protein-1 (SMN) [152]. Treatment of the patient-specific iPSC derived neural cells with valproic acid and tobramycin increased SMN protein levels suggesting that drug screening was possible in this system.

In another study by Marchetto and colleagues, hiPSC derived from a patient with Rett Syndrome, exhibited reduced spine density and neurons having smaller cell bodies [153]. Neurons derived from these hiPSCs were employed in a drug screen that identified IFG1 and gentamycin as being able to rescue synaptic defects.

Alzheimer's disease, a neurodegenerative disorder characterized by deposition of amyloid β peptide (Aβ) formed from the precursor protein by sequential cleavage with β- and γ-secretase, has also been modeled using patient-specific iPSCs [154,155]. These hiPSCs were differentiated into neuronal cells expressing the forebrain marker, FOXG1 and neocortical markers, CUX1, SATB2, CTIP2, and TBR1 as well as amyloid precursor protein, β-secretase and γ-secretase components [155]. Differentiated cells were capable of secreting Aβ into the conditioned medium. Aβ production was inhibited by β-secretase and γ-secretase inhibitors, as well as sulindac sulfide, a non-steroidal anti-inflammatory drug [155]. Therefore, patient-specific iPSC modeling of Alzheimer's disease is feasible and testing of candidate drugs with this system is possible [154,155].

Cooper and colleagues derived hiPSC from patients with Parkinson's disease with a mutation in *PINK1* (PTEN-induced putative kinase 1) and *LRRK2* (leucine-rich repeat kinase 2) genes. These hiPSCs were differentiated to neural cells and analyzed for mitochondrial function, including production of reactive oxygen species, mitochondrial respiration, proton leakage, and mitochondrial movement [156]. Mitochondrial dysfunction in hiPSC-derived neural cells from familial Parkinson's disease patients and at-risk individuals could be rescued with coenzyme Q(10), rapamycin, and the LRRK2 kinase inhibitor GW5074 suggesting that pharmacological rescue of mitochondrial deficits in these cells may result in a viable treatment option.

Drawbacks and Potential Limitations

Diseases are divided into three types, monogenetic diseases caused by a single mutation; multigenic diseases, caused by mutations in multiple genes and diseases caused by environmental factors. In addition, there are multiple diseases with more complex causalities involving interactions of genetic and environmental components. While patient-specific iPSC can model monogenetic diseases with a defined phenotype, multifactorial diseases require multiple tissue types and, thus, will be more challenging. Additionally, some diseases occur in late adulthood and would presumably require lengthy culture conditions for hiPSC modeling and even then the modeling may not represent true pathological conditions [157]. Also, incomplete target cell enrichment following differentiation is a problem that must be overcome [158] (see also above). Clonal variation of hiPSCs is

also a problem because it requires generation and testing of at least several clones from each patient and comparison of phenotypes and differentiation potential of each clone. Moreover, the lesion ultimately responsible for a disease phenotype may manifest at a particular stage of differentiation. Therefore, effective drug screens will need to measure effects during the entire differentiation process. Currently, this is not feasible. Finally, adult manifested lesions may not be amendable to modeling using current iPSC technology due to the current inability to generate truly mature cell types from iPSCs (see above).

Regenerative Medicine

In principle, patient-specific iPSC are preferable to hESCs due to several reasons. First iPSC generation is not fraught with the ethical dilemma surrounding ESC generation [159,160]. Second, transplantation of allogenic ESC-derivatives requires immunosuppressive therapy and constant surveillance to prevent cell/tissue rejection [161]. In theory, patient-specific iPSC-derived cellular transplantation should avoid the immune response since these cells are autologous. However, three issues still need to be addressed. First, although iPSC and ESC share most, if not all, functional properties, there have been reports of at least partially persistent epigenetic memory [111,162-166]. This might skew iPSC differentiation potential towards certain lineages. Second, there have been several reports that a variety of genetic lesions accrue during the reprogramming process [167-173]. The generality of these observations and whether they are a function of the specific reprogramming technologies are two open questions. A consensus will also have to be reached as to what is the acceptable level of potential genetic changes, in general, as well as for specific hiPSC applications. Finally, there have been reports from the murine system, that iPSCs may be immunogenic, even in syngeneic contexts [174]. When these issues are resolved, strategies for replacing cells that have died off in diseases such as Parkinson's, or cells that do not produce proper proteins or factors such as in Hemophilia might be ideal targets for hiPSC-based approaches. In addition, the prospect of correcting a gene mutation in patient-specific iPSCs and then transplanting the corrected differentiated somatic cells back into the disease-affected individual is tremendous.

Although iPSC-based therapies in humans are not yet feasible, several proof-of-concept studies in animals show promise. One of the first studies on iPSC therapy was accomplished by Wernig and colleagues using a rat model of Parkinson's disease [175]. Mouse fibroblasts were reprogrammed into iPSCs and then differentiated into neuronal precursors. These cells were then sorted for neuronal markers and injected into adult rats suffering from drug induced Parkinson's disease. The transplanted cells were shown to engraft into the adult rat brain and form axonal connections with native neurons. Furthermore, some functional dopaminergic neuronal recovery was observed in rats injected with iPSC-derived dopaminergic cell. In another study, Xu and colleagues injected the livers of irradiated hemophilia A mice with iPSC-derived endothelial cells that produced wild-type factor VIII (mutated in Hemophilia A patients) [176]. A bleeding assay determined that all transplanted mice survived due to the presence of functional Factor VIII. In another case, functional recovery of spinal cord injury was demonstrated by Tsuji and colleagues using normal iPSC-derived neurospheres that were transplanted into the brain of a spinal cord injury mouse model [177]. The mice exhibited functional re-myelination and axonal re-growth as well as differentiation of neurospheres into all three neuronal cell types including neurons, astrocytes and oligodendrocytes. As mentioned previously, correction of a disease-causing mutation has been demonstrated by Hanna and colleagues, who employed a humanized sickle cell anemia mouse model where iPSC bearing the sickle mutation were corrected by gene specific targeting [102]. Mice transplanted with hematopoietic progenitors derived from the corrected iPSCs exhibited restored hemoglobin function. Certainly the promise of regenerative therapies employing iPSCs or their derivatives are evident from these early studies. However, further studies must be done to address the safety of iPSC-based cell therapeutics before it can be transitioned into the clinic [178].

Precision Personalized Medicine

Precision personalized medicine describing the diagnostic, prognostic and therapeutic strategies precisely tailored to an individual patient's requirements is the future of effective patient treatment. This concept was predicted in the late 1800's by Canadian physician Sir William Osler who noted in 1892 "If it were not for the great variability among individuals, medicine might as well be a science, not an art." [179]. Although medicine will very much remain an art, the medical community is beginning to understand the role of inter-individual variability on patient therapy.

A modern and evolving area is to integrate personal genetic and genomic information, clinical patient assessment, family history and medical advances in order to tailor clinical therapeutics according to an individual patient's needs. This holistic, integrative approach is known as pharmacogenetics, if in reference to germ-line encoded differences in metabolic enzymes responsible for drug deposition. If in reference to the broader application of genomic technologies to drug discovery, efficacy and toxicity it is called pharmacogenomics. Current research interests include identification of the genetic basis of diseases, studying how genes and the environment interact to cause or influence the progression or severity of human diseases, and identification and application of pharmacogenetic biomarkers to facilitate more effective drug therapies. We refer the interested reader to the following literature for more detailed insight into these topics [179-181].

In the era of GWAS, where scientist and clinicians compile data on polygenetic linkage to complex multifactorial disorders, patient-specific iPSCs can provide a new model to explore the mechanisms of how polygenic modifications identified by GWAS contribute to disease pathology [142]. In addition, patient-specific iPSC-based pharmacogenomics, i.e., genomic and epigenomic profiling of disease-relevant cell types derived from patient-specific iPSCs can provide invaluable synergistic information to classical GWAS. When the gathered knowledge is coupled with drug databases and/or hiPSC-based drug discovery screens, and the patient's clinical health information it may help predict the most effective and safest pharmaceutical therapy for the individual patient.

In conclusion, the combination of established clinical-pathophysiological indexes, GWAS, patient-specific iPSC disease modeling, drug evaluation, discovery and development, as well as hiPSC-based pharmacogenomics has the potential to unravel the causative or predisposing genetic loci of diseases ultimately resulting in improved diagnostic predictors, earlier detection and finally the development of individual patient-tailored therapeutics. This is true precision personalized medicine.

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