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Induced pluripotent stem cells: methods, disease modeling, and microenvironment for drug discovery and screening

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Abstract: The raging ethical, religious, moral, political, and safety issues pertaining to the use of embryonic stem cells for therapy and research have prompted researchers to develop possibly better alternatives that are more efficacious and serve multiple purposes. Induced pluripotent stem cells (iPSCs) developed by multiple methods provide an unparalleled source of cells that could be differentiated into progeny cells. In cell-based disease models the progeny cells mimic, at least in part, the pathology of the studied disease and are used to improve the mechanistic understanding of the pathology. Furthermore, these cells could be used to test the efficacy and safety of drugs that can reverse this phenotype. This review provides an overview of the aforesaid methods for iPSC generation and the development of certain disease-based model systems. In this review, the focus is on model systems for neurological and hematological diseases. Last but not least, the importance of the microenvironment is underscored and the significance of these model systems is discussed in the context of drug screening and development.

Key words: Induced pluripotent stem cells, reprogramming methods, integrating/nonintegrating, induced pluripotent stem cell disease models, neurological/hematological diseases, induced pluripotent stem cell microenvironment modeling, drug discovery/screening

1. Introduction

There are raging ethical, religious, moral, political, and safety issues pertaining to the use of embryonic stem cells for therapy and research. Moreover, the known variations, including epigenetic factors, involving mouse stem cells have led researchers to develop alternative methods to obtain stem cells/stem cell-like cells. The Nobel Prize-winning effort of Shinya Yamanaka and his coworkers involved the reprogramming of mouse skin fibroblasts using retroviral-mediated transduction of genes encoding for transcription factors (TFs). However, the limitations pertaining to the extent and efficiency of reprogramming as well as the possible promiscuous integration and tumor formation have led researchers to refine the existing methods (e.g., integration-free plasmid-based approaches) and include chemicals that can possibly supplement and/or replace the pivotal 4 TFs. Efforts are also ongoing to develop and refine cell lines and reagent cocktails that are intermediate in terms of their epigenetic state, between the fully differentiated state and the development of stem cell-like cells (induced pluripotent stem cells (iPSCs)). In all of these cases, easily accessible and sampled autologous fibroblasts have been the cell type of choice in the development of disease models. Such models should mimic the microenvironment and (at least in part) the mechanisms of pathogenesis. This approach provides an opportunity for the testing of drugs and chemicals, including nanomaterials of biomedical and environmental significance. In addition, these stem cells should satisfy the criteria for pluripotency and should be positive for the stem cell markers characteristic of the iPSC phenotype. This review will also discuss the state-of-the art methods (ex vivo and in vivo) to establish and maintain iPSCs. Moreover, the focus will be on both disease modeling and drug discovery and testing.

2. Currently available methods for reprogramming cells to produce iPSCs

Somatic cell nuclear transfer (SCNT), a technique pioneered by Gurdon et al. (1958), involved the transfer of a mature cell's nucleus into an enucleated egg; this egg was eventually transformed into a living frog. Other researchers showed that the epigenome of somatic cells (adult thymocytes) could be reprogrammed by their fusion with embryonic stem cells, inducing these somatic cells to exhibit pluripotency in vivo (Tada et al., 2001). Further refinements to improve the efficiency of reprogramming involved fractionation of pluripotent stem cell-like cells

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from the differentiated cells. The nuclei from these cells were able to reprogram oocytes at a higher efficiency (Pan et al., 2015) and provided corroborative evidence for the fundamental idea that the relatively undifferentiated cells had a better reprogramming efficiency (Oback and Wells, 2007). These findings were also consistent with a study wherein it was demonstrated that iPSC generation could be enhanced (approximately eightfold increased) from a subpopulation of SSEA3+ human dermal fibroblasts (Byrne et al., 2009). Several methods are currently available to reprogram adult cells and convert them to a stem cell state that resembles that of embryonic stem cells. One of the methods used often involves retroviral-mediated transduction of TFs. These factors, used singly and/or in combination with chemicals like Valproic acid (Huangfu et al., 2008), have been used to dedifferentiate cells including fibroblasts, neural stem cells, stomach cells, liver cells, keratinocytes, amniotic cells, blood cells, and adipose cells in order to produce stem cell-like cells (Robinton and Daley, 2012). Their efficiency is low (0.001%–1%) and the reprogramming is not complete. The other disadvantages include a slow rate of conversion, nonspecific genomic integration with the possibility of activating a latent oncogene, and incomplete proviral silencing. Takahashi and Yamanaka's (2006) ground-breaking research findings involved the initial screening of 24 TFs for their reprogramming potential using a combination of reporter gene technology and a neomycin resistance marker. The resistance marker enabled the identification of cells with the embryonic stem cell-like phenotype in which reactivation of the embryo-specific genes had occurred. Their experiments finally culminated in the identification of 4 TFs (Oct-4, Sox-2, Klf-4, and c-Myc (OSKM)), which, when transfected in combination, could reprogram mouse embryonic fibroblasts (MEFs). Such cells were isolated from $Fbx^{\beta neo/\beta neo}$ embryos and were stem cell-like cells. However, their efficiency of conversion was relatively lower. Their stem cell phenotype was inferred based on morphology and marker gene expression; they developed tumors following injection into nude mice and into blastocysts for the development of germ layers (Takahashi and Yamanaka, 2006). Subsequently, the same 4 factors were used to reprogram human adult fibroblasts with the aim to generate stem cells that could be used as patient-specific disease models. They were demonstrated to be embryonic stem cells in terms of their shape, proliferation potential, surface protein profile, gene expression, epigenetics of the pluripotent cell-specific genes, activity of the telomerase enzyme, and the ability to form the germ layer (Takahashi et al., 2007). Another study provided corroborative evidence for the role of ectopic expression of the aforesaid 4 TFs in the reprogramming of dermal fibroblasts; these reprogrammed cells resembled those of the stem cell lineage in terms of morphology, gene expression profile, and karyotypic stability (Lowry et al., 2007). In both cases, the ability to form all 3 germ layers was demonstrated. Integrating lentiviral vectors have also been used to reprogram human somatic cells using a different combination of the 4 TFs (OCT4, SOX2, NANOG, and LIN28). The stem cells produced thereafter exhibit a gene expression profile, surface antigenicity, karyotype, and telomerase activity that are characteristic of human embryonic stem cells. They have the capability to develop into highly differentiated derivatives of all 3 primary germ layers. This may be the method of choice, provided mutation as a result of viral integration is avoided (Yu et al., 2007). Subsequently, lentiviral vectors were genetically modified to conditionally express Oct-4, Sox-2, Klf-4, and c-Myc under the control of the doxycycline-inducible reverse tetracycline transactivator (rtTA) (Tet-On system). These vectors were transfected into a 2-day explant culture of mouse pancreatic β cells (Rat Insulin Promoter (RIP)-Cre/LacZ). After 24 days, an expansion in 80% of the colonies was observed with activation of the pluripotency markers. Reprogramming efficiency ranged from 0.1% to 0.2% and colony expansion was done in the absence of doxycycline. The reprogrammed progeny from terminally differentiated cells expressed markers characteristic of pluripotent cells, formed teratomas, and was capable of germ line transmission in chimeric animals (Stadtfeld et al., 2008a). This doxycycline-inducible lentivirus system was extended to improve the efficiency of reprogramming in both primary and secondary hiPSCs (from human fibroblasts and keratinocytes), unlike the viral-mediated transduction (took several weeks for human keratinocytes) (Maherali et al., 2008; Stadtfeld et al., 2008b). A single mouse lentiviral cassette-based system combining the mouse transcripts for all 4 factors using a 2A peptide and an internal ribosome entry site in postnatal mouse tail-tip fibroblasts was a method that represented a significant milestone in the reprogramming field. The success of this method was determined based on morphology, markers, ability to form germ layers in teratoma assays, and chimera formation (Sommer et al., 2009). Another group of researchers used a similar approach to introduce human cDNAs for 3 TFs (Oct4, Sox2, and Klf4) into adult dermal fibroblasts from a humanized version of a mouse model system for sickle cell disease, using a self-inactivating lentiviral (SIN) vector. These genes were downstream of the elongation factor 1α promoter with a porcine virus teschovirus-1 2A sequence (ribosome skipping) between them. After 30 days, the colonies exhibited markers for pluripotency (e.g., endogenous Oct4, Sox2, NANOG, alkaline phosphatase, and stage-specific embryonic antigen-1 (SSEA-1)) and were able to produce teratomas in immunocompromised

mice and chimeras in murine blastocysts. The inserted vectors (3 of them) could be excised with the Cre/Lox recombinase methodology and the LoxP site did not affect coding, sequences, promoters, and regulatory elements (Chang et al., 2009). Subsequently, a similar humanized version of this single cassette lentiviral vector system was developed for human cells. This system also had the advantage of excising the transgenes subsequent to reprogramming, due to the presence of a cleavable integration site, apart from regulating TF expression. This methodology was extended to skin fibroblasts from human donors afflicted with various types of lung diseases (cystic fibrosis, emphysema related to α-1 antitrypsin deficiency) apart from scleroderma and sickle-cell disease. Disease-specific iPSCs that had the potential to produce tissues that were endodermal in origin were generated (Somers et al., 2010). This method provides an approach for the controlled expression of TFs, in addition to enabling their transduction into dividing and nondividing cells. However, genomic integration and the requirement of transactivator expression are limitations of this method. The issue of promiscuous, potentially damaging, vector integration-mediated mutational events have been circumvented by the use of nonintegrating lentiviral systems and have been adopted in currently ongoing clinical trials for the treatment of various diseases (Kumar et al., 2011). These vectors circumvent the problem of viral or plasmid-mediated integration of genes encoding for the reprogramming TFs in the host genome, thereby increasing the risk of malignant transformation. Adenoviral vectors have been used in both mouse and human systems. In mouse systems (fibroblasts and liver cells) nonintegrating adenoviruses can be used to introduce OSKM factors and produces iPSCs. Characterization by measuring the DNA methylation pattern, marker analysis, teratoma formation, and formation of tissue of the germline lineage in chimeras also provided evidence for the generation of cells of the desired type (Stadtfeld et al., 2008c). In human embryonic fibroblasts (HEFs), adenoviral-mediated ectopic expression of these factors (c-Myc, Klf4, Oct4, and Sox2) produced 3 stable iPSC colonies (3/12) with the characteristic morphology and markers as evidence for their identity. There was no integration of the virus as demonstrated using Southern blotting and PCR. These cells could produce dopaminergic neurons in vitro. Teratoma formation in 5 weeks was also seen in nonobese diabetic/severe combined immunodeficiency (SCID) mice following subcutaneous (SC) injection of each of these cell lines (Zhou et al., 2009). In addition to nonintegrating adenoviral systems, extrachromosomal plasmids have been used. One plasmid carried cDNAs for OSK TFs, while the other had the c-Myc oncogene. These plasmids were transfected into MEFs to generate iPSCs which could produce teratomas and chimeras in appropriate test systems. Most importantly, there was no evidence of viral integration (Okita et al., 2008). A variation of this experimental design with 4 plasmids, each harboring one of the 4 key TFs (Oct-4, Sox-2, NANOG, and Lin-28 (OSNL)), was used for their transient expression. Since no packaging vectors were used, the risk of generating virions was circumvented. Moreover, there was no need for subcloning for the identification of cells without exogenous DNA or there were no stability/selection issues. There was no integration and the cells were pluripotent, formed teratomas, and were able to differentiate into cells that resembled those of hepatocyte and cardiac lineages (targeted differentiation) (Si-Tayeb et al., 2010). As an alternative strategy for the generation of iPSCs, Sendai viruses (a nonintegrating RNA virus) can be used. The resultant cells were pluripotent and their demethylation pattern was characteristic of cells of this type. Enrichment of those virus-free reprogrammed cells was accomplished by using antibodies that recognized and eliminated those cells that expressed a surface marker (HN) present on Sendai virus-infected cells. Subsequently, germ layer differentiation and targeted development into cells like beating cardiomyocytes, neurons, and bone and pancreatic cells were demonstrated (Fusaki et al., 2009). As an alternative virus-independent "simplified" strategy to reprogram HEFs and produce stable iPSC lines, the PiggyBack (PB) transposon/transposase system was used to deliver the Dox-inducible TF payload to fibroblasts. Induced pluripotency was demonstrated by the expression of markers as well as the propensity of these cells to differentiate. Seamless excision was also demonstrated in murine iPSC lines in which TFs were joined by 2A sequences. This method has an efficiency of 0.1% and no genomic integration was observed. However, several cell lines would have to be screened subsequent to the transfection (Woltjen et al., 2009). In a major step forward in terms of avoiding the use of both chemicals and genes, recombinant proteins for the 4 key TFs (OSKM) fused to a cell-penetrating peptide (CPP) were introduced into human fibroblasts. The resultant cells (iPSCs) were similar to human embryonic stem cells in terms of morphology and markers and were maintained for 35 passages. Furthermore, they were capable of germ layer formation in vitro and formed teratomas in vivo (Kim et al., 2009). The risk of introducing potential harmful virally-mediated genetic material was totally eliminated. In addition to its safety, this method is fast and avoids the time-consuming selection of potentially integration-free iPSCs. Lastly, research and/or industrial centers that have an established recombinant protein production system can use this approach to produce iPSCs from these "chemically-defined" reprogramming proteins in a cost-effective

manner (Zhou et al., 2009). Nonintegrating synthetic RNA has been used to efficiently differentiate RNA-iPSC pluripotent stem cells (RiPSCs) into mature myogenic cells. This approach is safer than viral/other genetic approaches and the efficiency is also higher (approximately 2%, which is better by two orders of magnitude). Embryonic stem-like colonies appeared in 17 days in contrast with the 4 week period for viral-mediated approaches. The in vitro transcript had a 5' guanosine cap and 5' (with a strong Kozak translational initiation signal sequence) and α-globin 3' untranslated region (UTR) to improve its half-life and its translational efficiency (Warren et al., 2010). This construct was shown to bypass the innate antiviral responses. The kinetics and efficiency of reprogramming was better (with a range of about ~1%–4.4%) without genomic integration. miRNA-based strategies can improve the efficiency of the process by two orders of magnitude. This reprogramming was demonstrated by the formation of iPSCs (induction of Oct4 expression) following the introduction of miRNA302 (miR302/367 cluster) in both mouse and human somatic cells. The efficiency of this reprogramming process was also dependent on the decreased expression of HDAC2 (Anokye-Danso et al., 2007). Another paper provided corroborative evidence for the involvement of specific microRNAs. In this regard, a combination of microRNAs (mir-200c plus mir-302s and mir-369s family of mature double stranded microRNAs) was involved in the reprogramming process. This method has the obvious advantage of not having to employ viral-based strategies (Miyoshi et al., 2011). A summary of this section is provided in Table 1.

3. Disease modeling using iPSCs: focus on neurological and hematological diseases

3.1. Neurological models

Amyotrophic lateral sclerosis (ALS) is a heterogeneous neurodegenerative disease that is progressive in nature and affects the motor neurons of the brain and spinal cord. Of the cases that exhibit the inherited form of the disease, 20% have a point mutation in the Cu/Zn SOD1 genes. There are transgenic rodents that carry the mutant human SOD1 genes with amino acid substitutions in G93A, G85R, and G37R. This model system mimics the clinical and histopathological features of the human disease (Julien and Kriz, 2006) despite the lack of suitable animal models for all types of this disorder. Furthermore, it has been observed that transplantation of stem cell-derived astrocytes or astrocyte precursor cells can home in and restore the functional capabilities of this dysfunctional cell type in vivo (Nicaise et al., 2015). Due to their excellent therapeutic potential, patient-specific motor neurons derived from iPSCs offer an excellent opportunity to be used as models for the different subsets of ALS. Moreover, this iPSC model can help in better understanding the relative contribution of the microenvironment (support cells) around the regenerating motor neuron. The fully defined iPSCs could also be used for drug testing and for generating autologous cells for therapy (Dimos et al., 2008; Richard and Maragakis, 2015). The membranes of iPSC-derived motor neurons from patients with ALS were hyperexcitable due to delayed rectifier potassium current. This pathology was corrected by using an activator of the potassium channel (retigabine) in terms of improving the survival of motor neurons with the mutations in the SOD1 gene (Wainger et al., 2014). Human fibroblast-derived iPSCs from ALS patients carried a TAR DNA-binding protein 43 (TDP-43) M337V mutation and they were differentiated into functional motor neurons. The relevance of this ex vivo system was underscored by the demonstration of the presence of similar mutations (mutations in transactive response DNA binding protein 43 kDa –TARDP) in patients with ALS-mediated neurodegeneration and misaccumulation of TDP-43. Such mutant neurons had a decreased survival and were more sensitive to chemical (LY294002)-mediated selective PI3K inhibition (Bilican et al., 2012). A motor neuron cellular model was developed from fibroblasts isolated from sporadic ALS patients. These neurons were able to mimic the pathology (TDP-43 protein aggregation) seen in vivo in postmortem tissue from one of the patients, which was the source of iPSCs (Burkhardt et al., 2013). Parkinson's disease (PD) is the second most common human degenerative disease with the loss of dopamine-producing neurons in the substantia nigra. It has been classified broadly into the familial and sporadic forms. In both cases the presence of a strong genetic component and the lack of a cure for this disease have prompted researchers to develop disease models in a dish. This was made possible subsequent to the differentiation of iPSCs into motor neurons in vitro to mimic, at least in part, the mode of pathogenesis of PD (Badger et al., 2014). An iPSC cell lines with a homozygous p.G2019S mutation in the leucine-rich repeat kinase 2 (LRRK2) gene was produced. Another cell line carried a full gene triplet repeat of the α-synuclein encoding gene and synuclein alpha was generated. These DA neurons from these two cell lines were considered to be good models for early PD (Byers et al., 2012). Mutations in the aforesaid gene for LRKK2 in the mitochondrial genome of a PD patient have been known to increase the susceptibility of iPSC-derived neural cells to DNA damage due to oxidative stress. Individuals that harbor homozygous or heterozygous LRRK2 G2019S mutations, or those individuals carrying the heterozygous LRRK2 R1441C (susceptibility marker) had higher levels of mtDNA damage than unrelated healthy individuals. The

Table 1. Currently available methods for reprogramming cells to produce iPSCs (from section 2 of this paper).

Sl. No.	Reprogramming method	Citation details
1	SCNT-transfer of a mature cell's nucleus into an enucleated egg	Gurdon et al., 1958
2	Cell fusion, fusion of adult thymocytes with embryonic stem cells, epigenome reprogramming, pluripotency in vivo	Tada et al., 2001
3a	Relatively undifferentiated cells, better reprogramming efficiency	Oback and Wells, 2007
3b	Fractionation of subpopulation of SSEA3+ human dermal fibroblasts, iPSC generation could be enhanced (approximately eightfold increase)	Byrne et al., 2009
3c	Fractionation of pluripotent stem cell-like cells from the differentiated cells, improvements in reprogramming efficiency	Pan et al., 2015
4a	Reprogramming, combination of transcription factors + epigenetic modifier	Huangfu et al., 2008
4b	Dedifferentiation into stem cell-like cells, fibroblasts, neural stem cells, stomach cells, liver cells, keratinocytes, amniotic cells, blood cells and adipose cells	Robinton and Daley, 2012
5a	Identification of 4 TFs (Oct-4, Sox-2, Klf-4, and c-Myc (OSKM)), retroviral transduction, reprogramming mouse embryonic fibroblasts (MEFs)	Takahashi and Yamanaka, 2006
5b	Identification of 4 TFs (Oct-4, Sox-2, Klf-4, and c-Myc (OSKM)), retroviral transduction, human adult fibroblasts	Takahashi et al., 2007; Lowry et al., 2007
6	Different combinations of 4 TFs (OCT4, SOX2, NANOG, and LIN28), integrating lentiviral vectors, reprogram human somatic cells	Yu et al., 2007
7	Doxycycline-inducible reverse tetracycline transactivator (rtTA)-based lentiviral system, conditionally express Oct-4, Sox-2, Klf-4, and c-Myc, 2-day explant culture of mouse pancreatic β cell (Rat Insulin Promoter (RIP)-Cre/LacZ)	Stadtfeld et al., 2008
8	Doxycycline-inducible lentiviral system to convert primary human fibroblasts and keratinocytes into human induced pluripotent stem cells	Maherali et al., 2008; Stadtfeld et al., 2008
9	Single mouse lentiviral cassette-based system, mouse transcripts for all 4 factors using a 2A peptide as well as an internal ribosome entry site in postnatal mouse tail-tip fibroblasts, morphology, markers, ability to form germ layers in teratoma assays, chimera formation	Sommer et al., 2009
10	Introduce human cDNAs for 3 TFs (Oct4, Sox2, and Klf4) into adult dermal fibroblasts from a humanized version of a mouse model system for sickle cell disease, using a self-inactivating lentiviral (SIN) vector, genes downstream of the elongation factor 1α promoter and a porcine virus teschovirus-1 2A sequence (ribosome skipping) between them; after 30 days, colonies exhibited markers for pluripotency (e.g., endogenous Oct4, Sox2, NANOG, alkaline phosphatase, stage-specific embryonic antigen-1 (SSEA-1)); were able to produce teratomas in immunocompromised mice as well as chimeras in murine blastocysts; inserted vectors (3 of them) could be excised with the Cre/Lox recombinase methodology and the LoxP site did not affect coding, sequences, promoters, and regulatory elements.	Chang et al., 2009
11	A similar humanized version of this single cassette lentiviral vector system was developed for human cells. This system also had the advantage of excising the transgenes subsequent to reprogramming, due to the presence of a cleavable integration site, in addition to regulating TF expression. This methodology was extended to skin fibroblasts from human donors afflicted with various types of lung diseases (cystic fibrosis, emphysema related to α-1 antitrypsin deficiency) in addition to scleroderma and sickle-cell disease. Disease-specific iPSCs were generated and had the potential to produce tissues that were endodermal in origin.	Somers et al., 2010
12	The issues of promiscuous, potentially damaging, vector integration-mediated mutational events have been circumvented by the use of nonintegrating lentiviral systems and have been adopted in currently ongoing clinical trials for the treatment of various diseases.	Kumar et al., 2011

Table 1. (Continued).

13	Adenoviral vectors have been used in both mouse and human systems. In mouse systems (fibroblasts and liver cells) nonintegrating adenoviruses can be used to introduce OSKM factors and produce iPSCs. Characterization by measuring DNA methylation pattern, marker analysis, teratoma formation, and formation of tissue of the germline lineage in chimeras also provided evidence for the generation of the cells of the desired type.	Stadtfeld et al., 2008
14	In human embryonic fibroblasts (HEFs), adenoviral-mediated ectopic expression of these factors (c-Myc, Klf4, Oct4, and Sox2) produced 3 stable iPSC colonies (3/12) with the characteristic morphology and markers as evidence for their identity. There was no integration of the virus as demonstrated using Southern blotting and PCR. These cells could produce dopaminergic neurons in vitro. Teratoma formation was also seen in 5 weeks in nonobese diabetic SCID mice following SC injection of each of these cell lines.	Zhou et al., 2009
15	Extrachromosomal plasmids have been used. One plasmid carried cDNAs for OSK TFs, while the other had the c-Myc oncogene. These plasmids were transfected into MEFs to generate iPSCs that could produce teratomas and chimeras in appropriate test systems. Most importantly, there was no evidence of viral integration.	Okita et al., 2008
16	A variation of this experimental design with 4 plasmids each harboring one of the 4 key TFs (OSNL) was used for their transient expression. Since no packaging vectors were used, the risk of generating virions was circumvented. Moreover, there was no need for subcloning for the identification of cells without exogenous DNA or there were no stability/selection issues. There was no integration and the cells were pluripotent, formed teratomas that were able to differentiate into cells that resembled those of hepatocyte and cardiac lineages (targeted differentiation).	Si-Tayeb et al., 2010
17	As an alternative strategy for the generation of iPSCs, Sendai viruses (a nonintegrating RNA virus) can be used. The resultant cells were pluripotent and their demethylation pattern was characteristic of cells of this type. Enrichment of those virus-free reprogrammed cells was accomplished by using antibodies that recognized and eliminated those cells that expressed a surface marker (HN) present on Sendai virus-infected cells. Subsequently, germ layer differentiation and targeted development into cells like beating cardiomyocytes, neurons, bone and pancreatic cells were demonstrated.	Fusaki et al., 2009
18	As an alternative virus-independent "simplified" strategy to reprogram HEFs and produce stable iPSC lines, the PiggyBack (PB) transposon/transposase system was used to deliver the Dox-inducible TF payload to fibroblasts. Induced pluripotency was demonstrated by the expression of markers and the propensity of these cells to differentiate. Seamless excision was also demonstrated in murine iPSC lines in which TFs were joined by 2A sequences. This method has an efficiency of 0.1% and no genomic integration was observed. However, several cell lines would have to be screened subsequent to the transfection.	Woltjen et al., 2009
19	To avoid the use of both chemicals and genes, recombinant proteins for the 4 key TFs (OSKM), fused to a cell-penetrating peptide (CPP) were introduced into human fibroblasts. The resultant cells (iPSCs) were similar to human embryonic stem cells in terms of morphology and markers and were maintained for 35 passages. Moreover, they were capable of germ layer formation in vitro and formed teratomas in vivo.	Kim et al., 2009
20	The risk of introducing potential harmful virally-mediated genetic material is totally eliminated in this method. Apart from its safety, it is faster and avoids the time consuming selection of potentially integration-free iPSCs. Lastly, research and/or industrial centers that have established recombinant protein production systems can use this method to produce iPSCs from these chemically-defined reprogramming proteins in a cost-effective manner.	Zhou et al., 2009

Table 1. (Continued).

21	Nonintegrating synthetic RNA has been used to efficiently differentiate RNA-iPSC pluripotent stem cells (RiPSCs) into mature myogenic cells. This approach is safer than viral/other genetic approaches and the efficiency is also higher (approximately 2%, better by two orders of magnitude). Embryonic stem cell-like colonies appeared in 17 days in contrast with the 4 week period for viral-mediated approaches. The in vitro transcript had a 5' guanosine cap, 5' (with a strong Kozak translational initiation signal sequence) and α-globin 3' UTR to improve its half-life and its translational efficiency. This construct was shown to bypasses innate antiviral responses. The kinetics and efficiency of reprogramming was better (with a range of about ~1%–4.4%) without genomic integration.	Warren et al., 2010
22	miRNA-Based strategies can improve the efficiency of the process by two orders of magnitude. This reprogramming was demonstrated by the formation of iPSCs (induction of Oct4 expression) following the introduction of miRNA302 (miR302/367 cluster) in both mouse and human somatic cells. Efficiency of this reprogramming process was also dependent on the decreased expression of HDAC2.	Anokye-Danso et al., 2007
23	Another paper provided corroborative evidence for the involvement of specific microRNAs. In this regard, a combination of miRNAs (mir-200c plus mir-302s and mir-369s family of mature double stranded microRNAs (miRNAs)) was involved in the reprogramming process. This method has the obvious advantage of not having to employ viral-based strategies.	Miyoshi et al., 2011

zinc finger nuclease (ZFN)-mediated targeted correction of the LRKK2 G2019S mutation in iPSCs reversed the damage in the mitochondrial DNA. The damage was undetectable in differentiated neuroprogenitor and neural daughter cells (Sanders et al., 2014). Modeling of late-onset disease (e.g., PD) requires changes in iPSCs (embryonic stem cell-like cells) that can mimic the disease phenotype in a better manner, especially in the context of aberrations in mechanisms pertaining to reactive oxygen species (ROS) levels (Campos et al., 2014). Furthermore, the introduction of the progerin gene (a truncated form of LMNA) in fibroblast-derived iPSCs helped in the manifestation of cell-based aging-related markers (Müller et al., 2012). An improved understanding of the signal transduction pathways can help in refining the protocols for enhancing the efficiency and extent of reprogramming. Comparison of the protocols for the BMP/TGF-β-mediated signal inhibition versus FGF2 signaling has provided mechanistic details that can serve to enhance the reprogramming process in human umbilical cord blood-derived iPSCs. Specifically, the former pathway is better than the latter in terms of the percentage of tyrosine hydroxylase neurons and the dopaminergic induction of these stem cells. Furthermore, 3 weeks after transplantation, proliferating cells with a tumor-like outgrowth in 2 out of 4 animals were observed when the FGF-2 signaling-mediated neural induction protocol was used (Effenberg et al., 2015). While it is widely accepted that iPSCs offer an unprecedented opportunity to develop patient-specific disease models and help in drug testing, the possibility of a few undifferentiated iPSCs becoming tumorigenic has led researchers to develop transdifferentiation-based strategies (lineage-specific reprogramming) (Lopez-Leon et al., 2014).

3.2. Hematological models

A protocol was developed wherein skin biopsies were taken from a patient with Fanconi anemia (FA). Subsequently, the defect was corrected in human fibroblasts and/or keratinocytes following which viral transduction of TFs (Oct4, Sox-2, and Klf-4) was performed. This method can be extended to treat other disorders amenable to gene-corrected reprogrammed iPSCs (Raya et al., 2010; Focosi et al., 2014). Mechanistically, reprogrammed cells exhibited activation of aberrant signals in the FA pathway (involved in genome maintenance and its protection against cross-linking agents). Such molecular events may lead to DNA-double strand breaks, senescence, and a decrease in reprogramming efficiency in both murine and human cells. However, genetic complementation of the defect can result in cells with a normal karyotype with a restoration of reprogramming efficiency and reduction in the senescent phenotype. These cells also undergo differentiation into cells of the hematopoietic lineage (Müller et al., 2012; Rio et al., 2014). Apart from the genes in the FA pathway, Brca2 protein has also been implicated in DNA repair processes, specifically homology-redirected recombination. Brca2 gene complementation in MEFs aided in the generation of iPSCs with a genetically stable, disease-free phenotype and could be differentiated into hematopoietic cells despite

having a relatively lower efficacy and limited engraftment potential. Apart from these limitations, other genetic alterations, not observed in the uncorrected parental cells, were detected by karyotyping and comparative genome hybridization (CGH) (Navarro et al., 2014). A more recent strategy, possibly for the first time, involved the use of ZFNs and nonintegrating lentiviral vectors with resultant improvements in the specificity and efficiency of gene targeting (40% after 42 days) in the AAVS1 safe harbor locus in fibroblasts from FA patients. Moreover, the genetically corrected reprogrammed cells were differentiated to produce disease-free hematopoietic stem cells (HSCs) (Liu et al., 2014). Disease modeling was made possible by the generation of FA cell lines from human embryonic stem cells and iPSCs. It was possible to identify a defect earlier in the heamangiogenic progenitors and hence, such iPSCs improved our mechanistic understanding, at least, in part, of the FA pathology (Suzuki et al., 2015) and underscored the importance of certain proteins in the reprogramming process (Yung et al., 2013). Moreover, these cell lines provided a system for the testing of agents that promoted hematopoietic differentiation. Furthermore, these cell lines could be used to test agents that rescued FA cells and converted them into cells with the normal phenotype (Liu et al., 2014). Shwachman–Bodian–Diamond syndrome (SBDS) is an autosomal recessive syndrome. This syndrome is associated with enzyme-mediated autodigestion (Kelley et al., 2013) and is linked to defects in the differentiation of pancreatic and hematopoietic cells. iPSCs generated from such patients were known to show increases in apoptotic phenomena and increased secretion of proteases in culture supernatants. This extracellular protease increase was shown to be linked to the deficits in exocrine pancreatic and hematopoietic differentiation in vitro since restoration of the normal phenotype could be accomplished by SBDS gene expression or by the use of protease inhibitors (Tulpule et al., 2013). A combination of reprogramming methods to generate iPSCs and gene targeting using lentiviral vectors has resulted in the development of models demonstrating their curative potential. This approach has been extended successfully to clinical trials. However, certain areas like stoichiometry between endogenous and transgenic hemoglobin (since β globin gene mutations occur at different sites) and safety due to the possibility of insertional mutagenesis and myeloablation-mediated toxicity should be focused upon to improve the efficacy and safety of this therapeutic strategy for correction of this monogenic disease in sickle cell anemia patients (Dong et al., 2013). Patient-derived iPSCs with 2 mutated beta globin alleles were obtained. The heterozygous condition, with one copy of the gene corrected, was obtained in a 2 step procedure. The first step involved the use of a plasmid encoding a drug resistant gene flanked by LoxP sites. Precise homologous recombination (HR) with ZFNs ensured the conversion/correction of one allele of the β globin gene. The second step involved the Cre recombinase excising the cointegrated selection gene in the intronic region, thereby reversing the suppressed gene expression at the transcriptional level. Gene expression was around 25%–40% following erythrocyte differentiation in comparison with the wild type levels (Zou et al., 2011). A publicly available open source approach (the oligomerized pool engineering method) was adopted to identify 3 ZFNs that could increase the hitherto reported decrease in HR frequency in iPSCs from sickle cell anemia patients. Such ZFNs enabled the transgene-free correction of a monogenic disorder mutation. The resultant cells were fully pluripotent and had a normal karyotype following removal of the reprogramming factors and the selectable drug resistant gene markers (Sebastiano et al., 2011). Advances in gene editing have been adapted to precisely correct such errors in the DNA sequence in iPSCs. Success also depends on gene expression being regulatable in a cell-specific condition-dependent manner (Jazwa et al., 2013).

β-Thalassemia is a common genetic disease due to mutations or deletions in the β-globin gene. Research is ongoing in the area of gene therapy since it is a promising approach for treatment of this disorder (Finotti et al., 2015). A study performed in China demonstrated HR-mediated hemoglobin subunit beta (HBB) gene correction in iPSCs derived from human fibroblasts isolated from a 2-year old β-thalassemia patient. Specifically, the gene corrected was a 41/42 deletion in the human globin gene. The corrected cells were able to produce normal human β globin in a mouse model (Wang et al., 2012). iPSCs derived from β-thalassemia could be genetically corrected with lentiviral-mediated gene transfer and it was found that the integration of the vectors occurred at common sites (non-randomly) for iPSCs produced from both isogenic and nonisogenic patients. No oncogene was selectively activated (Tubsuwan et al., 2013). More recently, advances in genome editing using transcription activator-like effector nucleases (TALEN)/PB or clustered regularly interspaced short palindromic repeats (CRISPR))/Cas9-based tools have served to improve the precision of gene targeting with minimal or no off-shore target effects. It has been shown that TALEN with a PB transposon vector could produce corrected cells (intron 2 mutation site IVS2-654) at a higher homologous gene frequency in comparison with the CRISPR/Cas9. Furthermore, β globin gene transcription was higher in the differentiated erythroblast progeny of such iPSCs using a OP9 coculture system (Xu et al., 2015). Demonstration of the precision of the gene targeting approach required the analysis of DNA sequences. Hence, both alleles of the HBB gene were

corrected by the ZFN/Cre recombinase in iPSCs from amniotic cells. The genetically modified cells were subject to CGH and whole-exome sequencing. In both steps (iPSC generation and gene targeting), substantial, but different genomic variations were observed. Hence, it has to be ensured that such genetic changes do not have adverse therapeutic consequences (Ma et al., 2015). Another report has highlighted the utility of the CRISPR/Cas9 system in correcting the HBB gene in the generated iPSCs. These iPSCs had a normal karyotype and were fully pluripotent. ROS formation was decreased and they exhibited a better differentiation potential (Song et al., 2015). The Yamanaka approach of reprogramming fibroblasts was adopted to generate iPSCs from bone marrow stromal cells from β-thalassemia patients. The gene expression profile resembled that of hESCs following lipofection of mRNA for TFs (OSKM +Lin28). However, the low hematopoietic potential (10% & CD34+) and a decreased colony forming ability warranted the need for further improvements in the protocols for optimized patient-specific autologous iPSCs (Varela et al., 2014). Another group provided further evidence for the CRISPR/Cas9/PB transposon system being an effective gene editing tool in terms of restoring HBB expression in iPSCs from β-thalassemia patients. No unwanted residual genetic footprint was observed in these patient-derived stem cell-like cells (Xie et al., 2014). Again, TALEN-based universal gene correction was demonstrated in integration-free β-thalassemia iPSCs in situ with no off-shore effects. The pluripotent iPSCs with a normal karyotype retained their differentiation potential as demonstrated by the formation of progenitor cells and erythroblasts cells of the hematopoietic lineage as well as by their expression of normal globin expression (Ma et al., 2013).

4. Microenvironment models for hiPSC

Classical evidence for the role of the microenvironment in terms of elasticity/stiffness is provided by the observation that the beating of the cardiomyocytes is optimal when it is cultured on a matrix with an elasticity of 1–2 kPa. This elasticity mimics that of an E4 embryo. The cell-intrinsic mechanosensitivity observed with sparsely cultured cardiomyocytes was also observed by those differentiated cells produced from iPSCs (Majkut et al., 2013). Another line of evidence was provided by the use of 4% polyethylene glycol (PEG)–96% polycaprolactone (PCL) for culturing cardiomyocytes derived from iPSCs. Culturing cardiomyocytes in such a microenvironment resulted in improved contractility and mitochondrial function apart from the switch of the fetal troponin to the adult form (Chun et al., 2015). In order to confer better liver functions for a longer period of time in vitro, semiconductor-driven microfabrication tools have been used to precisely control the microenvironment (Davidson et al., 2015). A 10-day old culture of patient embryoid body-derived iPSCs on concave hydrogel structures resulted in these cells differentiating into the 3 germ layers and forming cavities (another sign of differentiation). These results provided evidence of the important role of the microenvironment mimicking that found around the developing embryonic stem cells in vivo (Hribar et al., 2015). Culture conditions were created wherein the microenvironment existing in the aorta-gonad-mesonephros (AGM) region was mimicked using a combination of approaches. They were embryonic stem cells grown on primary AGM explant cultures or on AM20-1B4, a stromal cell line. This resulted in the increased production of embryonic stem cell-derived HSC progenitor cells in both experimental systems (Krassowska et al., 2006). A biodegradable polymeric nanostructure was used to mimic the extracellular matrix niche and provided mechanical support for the optimal growth of iPSCs as a multilayered 3D spheroid culture. In the absence of this material, the cultures were converted back to the 2D monolayer. This approach has tremendous ramifications since it may aid in the coupling of signal transduction pathways involved in the formation of iPSC-derived organs (Alamein et al., 2014). The content of PEG on polymeric films was modulated to improve the human mesenchymal stem cells differentiating into osteoblasts (Vega et al., 2012). In certain cases, (e.g., spinal cord injury) among other factors, the complex microenvironment may limit the ability of the produced iPSCs to be successfully engrafted. For example, in an immune-suppressed animal model for contusion spinal cord injury, iPSCs derived neural progenitor cells could not restore functionality (Pomeshchik et al., 2014). Poly dimethyl siloxane (PDMS)-patterned substrates that were plasma treated and coated with vitronectin were developed. These modified substrates provided a better microenvironment for cell adhesion and expressed markers for the undifferentiated state for 7 days (Yamada et al., 2014). The microenvironment also includes cell-cell interactions and factors released from them. Reprogramming of fetal fibroblasts may be favored due to cell-cell contact and/or factors produced from ovarian cells (Lim and Gong, 2013). The same concept applies to the development of hepatocytes from iPSCs, which, when differentiated into hepatocytes, can be used as disease models. Moreover, they can be used to test drugs and can also be differentiated, subsequent to their genes being corrected in iPSCs. In this regard, the role of the microenvironment is pivotal (Subba Rao et al., 2013). It is worthy of mention that apart from the mechanical and biochemical microenvironmental cues, cardiac stimulation by electrical means served as a positive factor in enhancing the differentiation of iPSCs into cardiomyocytes in vitro (Dai et al., 2013). Paracrine

factors in the ischemic microenvironment enhanced new blood vessel formation and increased the survival of the introduced cardiomyocytes in mice with myocardial infarction. These results were obtained subsequent to the transplantation of endothelial cells produced from reprogrammed porcine stromal cells (Gu et al., 2012).

5. Drug discovery and screening: focus on neurological and hematological disorders

The differentiated cardiomyocytes and hepatocytes from patient-specific iPSCs can be used as cell-based drug testing models that can potentially resolve toxicity issues, thereby possibly reducing the attrition rate in drug testing during clinical trials. This aspect is relevant since 30% of the drugs fail due to their toxicity to vital organs like the heart or the liver (Singh et al., 2015). The same technology involved in iPSC generation could be applied to the generation of cancer stem cell-like cells. Such cells were tested with compounds (e.g., Withaferin A) that can inhibit the growth and/or eliminate these stem cells (Krishnan, 2015a). Such a strategy can be extended to the development of a high-throughput screening (HTS) platform for the testing of novel anticancer drugs that can specifically target cancer stem cells, an important contributor to the observed recurrences/relapses in cancer patients (Nishi et al., 2014). The iPSCs from the fibroblasts of familial Alzheimer disease (AD) patients were differentiated into neurons. These cells, with mutations in the presenilin genes, exhibited an increase in amyloid β42 secretion. Such cells could be tested for the development of promising drug candidates like the secretase inhibitors and modulators (Yagi et al., 2011). This cell-based stratification model also provides the drug hunter an opportunity to identify the right subset of patients (in both familial and sporadic AD) for which a particular drug may be effective (Kondo et al., 2013). In another example, iPSCs carrying mutations in the TDP-43 were generated. The mutant protein levels were increased in the iPSC-derived differentiated motor neurons. This biomolecule was insoluble in detergent. This protein was bound to a spliceosomal factor (SNRBP2). Further, the cytosolic aggregates and shorter neuritis were respectively similar to what was observed in the postmortem human ALS tissue and in a zebrafish model. Last but not least, this cell-based model system provided a proof-of-concept approach in terms of testing (anacardic acid, a histone acetyltransferase inhibitor) a compound that can reverse the pathology at the cellular level (Egawa et al., 2012). Apart from human stem cells, wild-type and mutant SOD1 mouse embryonic stem cells may be differentiated into motor neurons. Such differentiated cells may be used to screen and select compounds with the potential to reverse this form of motor neuronal cell death. It was reported that Kenpaullone was able to inhibit two kinases: glycogen synthase kinase-3 and hepatocyte progenitor kinase-like/germinal center kinase-like kinase; however, Olesoxime and Dexpramipexole failed to show activity (Yang et al., 2013). The human iPSC (hiPSC)-derived neurons from schizophrenia (SCZD) patients had alterations in glutamate receptor expression and PSD-95 protein levels. Their neurite connectivity was decreased with aberrations in both Wnt and cyclic adenosine monophosphate pathways. The importance of modeling this phenotype was demonstrated by their reversal using Loxapine (an antipsychotic) (Brennand et al., 2011). iPSCs from skin fibroblasts of a SCZD patient were made to differentiate into neural cells. These cells had higher ROS levels and their oxygen consumption extramitochondrially was also higher than comparable controls. The elevated ROS levels could be brought back to normalcy using Valproic acid, again validating the approach of developing differentiated disease models from autologous patient-specific cell based pathology models (Paulsen Bda et al., 2012).

Spinal muscular atrophy, an autosomal recessive disease, results in the loss of the α motor neurons in the spinal cord and is considered to be one of the leading genetic causes of mortality among infants. This loss has been attributed to a decrease in the level of survival motor neuron 1 protein (SMN1 protein) due to mutations in its gene (SMN). It has been reported that there are 16 drugs at various stages of preclinical and clinical drug development; other strategies involve upregulating the SMA gene using antisense oligonucleotide-based modulators (Cherry et al., 2014).

In iPSCs derived from β-thalassemia patients (mutation in β-41/42-17), novel Valproic acid derivatives have shown potential in the increased synthesis of fetal hemoglobin at concentrations that may possibly be close to the clinically relevant level (Rönndahl et al., 2006).

The iPSCs from individuals afflicted with Hutchinson–Gilford progeria syndrome exhibited a truncated farnesylated form of LMNA. The resultant LMNA C1824T mutant smooth muscle cell showed their contractile ability following treatment with Carbachol (Zhang et al., 2011). Mature pancreatic cell types could be produced following treatment of SBDS-deficient SDS-iPSC-1 cells with a cocktail consisting of aprotinin, broad spectrum protease, lipase, and a pan-caspase inhibitor from SBDS patients. Moreover, aprotinin was found to significantly reduce cell death (Tulpule et al., 2013). This rapidly advancing technology has been based on studies involving an improved molecular understanding of the development of iPSCs from relatively more differentiated cells (Krishnan, 2015b). Apart from their mechanistic relevance, it has ramifications in regenerative medicine and the development of small molecules. Such molecules

can be used in therapy, in addition to their role in augmenting and/or replacing genetic reprogrammers to develop these stem cell-like derivatives. Such approaches would require an improved molecular understanding of the reprogramming process.

6. Conclusion

The autologous patient-specific iPSCs generated from easily accessible, biopsied samples from humans provide scientists with an unparalleled opportunity to develop and study disease models to better comprehend the mode of pathogenesis. These iPSC-derived differentiated progenies may be useful disease models, provided the microenvironment is mimicked as closely as possible. Furthermore, such thoroughly validated models can be used for efficacy safety testing of drugs in the HTS format.

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