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Induced Pluripotent Stem Cells: Next Generation Cells for Tissue Regeneration

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Abstract

More than two decades of *in vitro* experimentation supported by the data from experimental animal studies in both small as well as large experimental animal models have culminated into multiple clinical studies worldwide to assess their regenerative potential. Although the data generated from these studies have only met with cautious response from the researchers, efforts are still underway with the hope to refine the different aspects of cell-based therapy approach to develop it into an effective routine therapeutic intervention. Besides others, search for a cell type with optimal characteristics remains an area of intense research. Pluripotent stem cells in general, and induced pluripotent stem cells in particular have gained special attention of researchers due to their ability to adopt a morphofuntionally competent phenotype. They are being considered as surrogate embryonic stem cells albeit without moral and ethical issues of availability and having better immunological acceptability. We provide a head-to-head comparison of ESCs and iPSCs and an overview of stem cell therapy approach converging on the observed advantages of pluripotent stem cells during pre-clinical and clinical studies.

Keywords

ESC, iPSC, Regeneration, Stem Cells, Transplantation

1. Introduction

In our pursuit to stay alive and healthy, human beings have been fighting disease since the dawn of time. Our ways have been constantly evolving and so do the options of therapeutic intervention to support the intrinsic repair mechanisms of the biological system during diseased conditions, but the breakthrough we witness today is

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unprecedented. Last three decades of medical research have witnessed the emergence of molecular and cellular medicine that has advanced the field from symptomatic intervention to confront the root cause of the disease process at cellular and molecular levels. The newer concept is simple yet technically challenging as it involves "repair, replacement or regeneration of cells, tissues or organs to restore the impaired biological functions" [1].

The regenerative capacity of the tissues and organs varies significantly in different animals. While planarian worms possess the regenerative capacity to compensate for loss of any of their tissues, and even have the competence to produce the entire animal from a single cell [2], *homo sapiens* are naturally less privileged when it comes to regenerative ability. Hence, in the clinical perspective, researchers have always wondered if we could harness the power of regeneration. Decades of research in characterization of stem cells and exploration of their capacity of multi-lineage differentiation to adopt morphofunctionally competent phenotypes have brought us closer to harnessing their regenerative capability. Our review of literature provides a direct comparison of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) besides providing a critical appreciation of the progress made thus far in the use of these cells in pre-clinical settings as well as in the clinical perspective for developing pluripotent stem cell-based therapeutic modalities.

Stem cells are discriminated from their somatic cell counterparts on the basis of two important characteristics: their capacity of unlimited undifferentiated self-renewal and the ability to differentiate into various cell lineages in response to appropriate set of cues [3]. In addition to these discerning features, pluripotent stem cells express transcription factors that are characteristic of primitiveness (e.g. Oct3/4, Sox2 and Nanong) [4], and undifferentiated cell markers [5]. Various schemes have been adopted to classify stem cells based on their competence to differentiate into different specialized cell types, tissue source and surface markers [6]. Based on potency features, stem cells can be totipotent, pluripotent, multipotent, or unipotent (**Table 1**). A totipotent cell can give rise to all embryonic and extraembryonic structures with the zygote being an archetypical example. On the same note, pluripotent stem cells derived from the inner cell mass (ICM) are capable of differentiating into all cell types from the three germ layers ectoderm, mesoderm, and endoderm. Unlike the previously described two cell types, both multipotent and unipotent stem cells exhibit much restricted capacity of differentiation. Adult stem cells found in almost all tissues *i.e.*, skeletal and cardiac muscles, adipose tissue, peripheral blood or bone marrow, belong to the later categories and incidentally are convenient in their acquisition [7].

The pioneering work of Yamanaka *et al.* has led to the ground breaking discovery that somatic cells can be reprogrammed to pluripotent status [8]. The results of the innovative strategy won the authors laurels of being Nobel laureates besides reinvigorating the interest in the use of pluripotent stem cells. Their decade long search for appropriate combination of transcription factors for reprogramming of somatic cells revealed that forced expression of a quartet of transcription factors including *Oct*4 (Octamer-4), *Sox*2 (*Sry* box containing gene 2), *Klf*4 (Krupple like factor-4) and *c-Myc*(c-myelocytomatosis) could successfully transform the mouse skin fibroblasts to pluripotent status (**Figure 1**). Since the inception of these data, various research groups have attempted to optimize the classical 4-factror protocol to enhance the efficiency of cellular reprogramming of somatic cells besides improving their safety for human application. Noticeable modifications in the classical protocol include attempts to replace and/or reduce the number of transcription factors [9]-[13]. Moreover, there is a shift from usage of integrating viral vector delivery systems to the application of non-integrating viral and virus-free methods to enhance the reprogramming efficiency and safety of iPSCs for human application [14]-[19]. A detailed account of the topic is provided elsewhere in the review.

Table 1. Head-to-head comparison of ASCs, ESCs and iPSCs.

Characteristics	ASCs	ESCs	iPSCs
Potency	Limited (multipotent or uni-potent)	Pluripotent	Pluripotent
Availability	Easy	Difficult	Easy
Ethical issues	Less problematic	More problematic	Less problematic
Immunogenicity	Less problematic	More problematic	Less problematic
Self-renewing period	Shorter	Longer	Longer
Teratogenicity	Less tumorigenic	Yes	Yes

ASCs: Adult stem cells; ESCs: embryonic stem cells; iPSCs: Induced pluripotent stem cells.

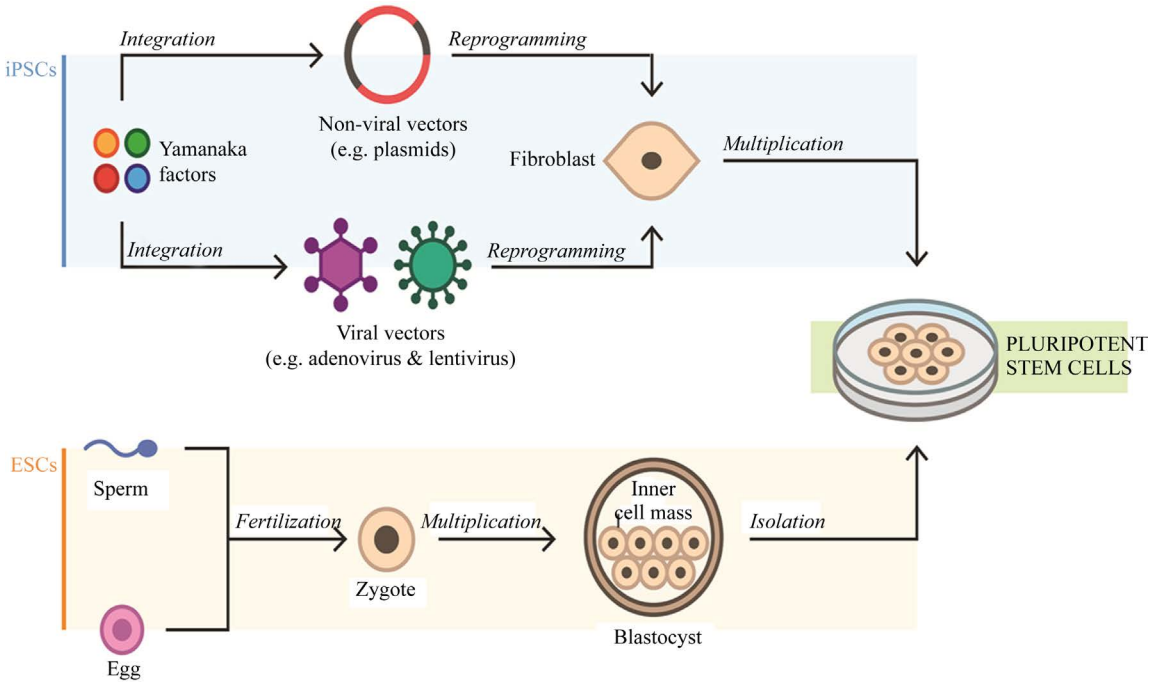

Figure 1. Isolation and production of ESCs and iPSCs.

2. Embryonic Stem Cells

Murine ESCs (mESCs) were first reported in 1981 in two back to back publications when the cells were identified as pluripotent with the capacity to form a chimeric animal as well as teratocarcinoma in an experimental immunodeficient mouse model [20] [21]. The cells were isolated from the *in vitro* cultures of the mouse blastocysts and showed conclusive evidence of multi-lineage differentiation. These data showed the feasibility of isolation of pluripotent cells from non-inbred embryos. Since these pioneering studies, multiple mESC lines from different research groups have been established and made available for further investigations (**Table 2**).

One and a half decades later, marking the advent of a new era in the field of medicine, Thompson *et al.* reported the first human ESC line using fresh or frozen cleavage stage embryos produced by *in vitro* fertilization for clinical use [22]. The authors reported 5 cell lines derived from 5 different embryos with all of the derivative cell lines showing normal karyotype. Typically, the cells exhibited distinctive features of an ESC including larger nucleus to cytoplasm ratio, high telomerase activity and continuous undifferentiated propagation *in vitro* culture for 5 - 6 months before cryopreservation. Moreover, the cell lines differentiated into cell types from all the three germ layers and formed teratomas when injected into immunodeficient mice. Until the writing of the manuscript, NIH website (http://grants.nih.gov/stem_cells/registry/current.htm) recorded 351 human ESC lines eligible for research use in the NIH funded projects and 34 ESC lines are under review and pending approval (http://grants.nih.gov/stem_cells /registry/pending.htm). Since the publication of the first report, hESC isolation and procurement protocols have been advanced to obtain clinical grade hESCs. Immunosurgery to isolate ICM from the blastocysts remains the most commonly employed method, however, the use of xeno-products during subsequent manipulations restricts the clinical application of the derivative cell lines [23]. Protocols based on mechanical manipulation and enzymatic digestion using combination of collagenase IV, dispase, and trypsin have also been reported [20] [24]. Although the use of enzymatic digestion requires less manipulation and can be efficiently applied on a large scale as compared to mechanical methods of isolation of hESC, use of enzymes causes genetic abnormalities in the developed cell lines [25]-[27]. In order to circumvent these methodological caveats, a laser beam-based system was reported to isolate ICM from trophoectoderm [28]. The technique was based on laser-assisted drilling of a hole in the zona pellucida and the isolated cells were plated in xeno-free culture conditions supported by the feeder cells. Despite these advancements, none of the studies published thus far has attempted to compare these protocols in terms of efficiency as well as the characteristic advantages of their respective derivative cell lines.

Table 2. ATCC Mouse (*Mus musculus*) ESC lines for research.

○ **G-Olig2**	#SCRC-1037™
○ **AB2.2**	#SCRC-1023™
○ **EDJ#22**	#SCRC-1021™
○ **ESF 158**	#SCRC-1016™
○ **B6/BLU**	#SCRC-1019™
○ **SCC#10**	#SCRC-1020™
○ **CE-1**	#SCRC-1038™
○ **RW.4**	#SCRC-1018™
○ **R1**	#SCRC-1011™
○ **R1/E**	#SCRC-1036™
○ **7AC5/EYFP**	#SCRC-1033™
○ **CE3**	#SCRC-1039™
○ **J1**	#SCRC-1010™

A direct comparison of mESCs and hESCs centred on the analysis of more than 400 genes has been reported [29]. Both cell types were cultured under standard set of conditions to support their undifferentiated status. The results revealed similarities in pluripotency determinant gene profile but showed absence of differentiation specific markers. Together, these data constituted a signature profile of a typically undifferentiated ESC. Despite having commonality in characteristics, both the cell types differed in their morphology, expression of markers such as stage specific embryonic antigen (SSEA)-1, SSEA4, vimentin, fibroblast growth factor-4 (FGF4) and trophoectoderm markers [29]. Moreover, the two cell types differed in their requirement of leukaemia inhibitory factor (LIF) for sustenance of self-renewal through high rate of cell proliferation and concomitant maintenance of undifferentiated pluripotency. Molecular mechanistic studies revealed that downstream of LIF receptor (LIF-R) and gp130 complex (both of which are hallmarks of mESCs), the activation of STAT3 significantly contributed in the undifferentiated self-renewal [30]. Although LIF independent self-renewal of hESCs remains their hallmark, there are reports that some mESCs may follow LIF independent pathway for their self-renewal without undergoing differentiation [31]. Experiments with both mESCs and hESCs have shown that transcription factor Nanog was expressed in ESCs as long the cells maintained their pluripotency irrespective of the cell type [32]. It is generally considered that endogenous Nanog has integral role in ESCs self-renewal in addition to the growth factor mediated stimulation of STAT3. A direct comparison of mESCs and hESCs is given in **Table 3**.

3. Culture and Differentiation Characteristics of ESCs

The most logical culture conditions for hESCs should mimic their natural habitat and microenvironment in the blastocyst to ensure their undifferentiated culture and propagation *in vitro*. The earlier studies frequently used mouse embryonic fibroblast (MEF) feeder layer as an essential component for *in vitro* cultures of ESC. The rationale behind the use of MEF as substrate was to enhance adhesion in the ESC and support their tightly packed 3-D growth similar to the embryo and to aid in nutrient provision [33]. Nevertheless, many applications of ESCs, especially their clinical compliance, necessitate optimized protocols to ensure culture conditions which are adherent to the ethical standards and in line with good manufacturing practices (GMP). Consistent with these prerequisites, feeder cells of human-origin including fibroblast feeder cells derived from fallopian tube epithelium, fetal foreskin, muscle, bone marrow, or amniotic epithelium have been successfully used as replacement of MEF [34]-[38]. Additionally, protocols for culturing hESCs have been developed in feeder-free conditions using matrigel and fibronectin. The hyaluronic acid (HA) hydrogel is so far the most superior matrix for maintaining undifferentiated hESCs, as it mimics the essential components of the ECM in the embryos such as bFGF, insulin, ascorbic acid, laminin, and activin-A, and also grants hESCs to retain their maximal differentiation capacity [39].

Table 3. Mouse ESC *vs* human ESCs.

	mESCs	hESCs
Morphology	Diverse	Rounded with sharp boundaries
***In vitro* culture requirements**	LIF inhibits differentiation	MEF or Human Feeder Cell Layer (recently, feeder-free culture is possible)[4]
Replication time	~12 hours	~36 hours[4]
Antigenic phenotype	SSEA-1	SSEA-3, SSEA-4[4]
Vimentin	Absent	Present
Trophoectoderm markers	Absent	Present
***β*-III tubulin**	Present	*β*-5 tubulin present
LIFR	High	Low/variable
Gp-130	High	Low/variable
FGF4	High	Absent
HRASP	Required	Pseudogene
E-hox	Required	No orthologue present
Fox-D3	Present/required	Low/absent
Lineage induction by Oct4	Mesoderm and Endoderm	Endoderm[2]
Lineage induction by SOX2	Ectoderm and Mesoderm	Not well-established[2] (it's importance for hESC is yet to be established as it is missing in some lines)[3]
NANOG's role in maintaining stem-cell-features	Pluripotency only	Pluripotency and self-renewal[2]

[1]Ginis I, Luo Y, Miura T, *et al*. Differences between human and mouse embryonic stem cells. *Developmental Biology*. 2004; 269(2):360-380. [2]Schnerch A, Cerdan C, Bhatia M. Distinguishing between mouse and human pluripotent stem cell regulation: The best laid plans of mice and men. Stem Cells. 2010; 28(3):419-430. [3]Bhattacharya B, Miura T, Brandenberger R, *et al*. Gene expression in human embryonic stem cell lines: unique molecular signature. Blood. 2004; 103(8):2956-296. [4]Gepstein L. Derivation and potential applications of human embryonic stem cells. Circ Res. 2002; 91(10):866-876.

Besides feeder layer, LIF remains an indispensable component of culture medium for undifferentiated maintenance of mESCs *in vitro* culture conditions [40]. However, significance of the presence of LIF for undifferentiated propagation of hESCs remains contentious. Molecular studies have shown that human LIF can induce activation of JAK-STAT3 signalling in hESCs however, these molecular events do not help hESCs in undifferentiated propagation [41]. When grown in non-adherent suspension culture conditions, ESCs spontaneously transform into globular embryoid bodies (EBs) containing ESCs differentiating to form cells from all the three germ layers. EBs can be induced to differentiate into various lineages, *i.e.*, neurons, cardiomyocytes, pancreatic *β*-cells, haematopoietic progenitors, muscle cells, endothelial progenitors, etc. by the addition of specific growth factors and ECM proteins (**Table 4**). For example, treatment with retinoic acid induces neuroectodermal differentiation of ESCs by initiating a cascade of intermediate unstable differentiation states *via* induction of the neuronal specific transcription factor Nurr-1 [42]. Similarly, treatment with nicotinamide increases the earliest cardiac-specific transcription factor Csx/Nkx2 that eventually forms the cardiomesoderm [43] [44]. ESCs can be used to study normal human embryonic development and pathological processes that allows researchers to extensively examine the normal/disease processes *in vitro*. These *in vitro* data can be extrapolated to treat incurable diseases such as Alzheimers, Parkinson's disease, diabetes, heart disease/failure, spinal cord injuries and

Table 4. *In vitro* differentiation of ESCs and iPSCs in to different cell lineages.

#	Experimental conditions	Cell type used	Tissue derived	Reference
1	All-trans retinoic acid	hESCs	Smooth muscle cells	*Huang et al.*, 2006
2	Co-culture with fetal liver stromal cells	hESCs	Functional erythrocytes	*Ma et al.*, 2008
3	Transient reactivation of c-Myc after reprogramming	Human dermal fibroblast derived hiPSCs	Megakaryocytes and Platelets	*Takayama et al.*, 2010
4	Sequential treatment with activin A + BMP4 + FGF2 + HGF	Fibroblast derived hiPSC	Hepatocyte-like cells	*Si-Tayeb K et al*, 2010
5	Mouse ESCs treatment with various concentrations of Simvastatin	Osteogenic Progenitors	Successful osteogenic differentiation	*Pagkalos et al.*, 2010
6	Pro-intestinal culture + activin + Wnt3A+ FGF4	hiPSCs and hESCs	Intestinal tissue	*Spence et al.*, 2011
7	Feeder free + FGF inhibitor: SU5402, MEK inhibitor: PD184352, and GSK3 inhibitor: CHIR99021)	Rat ESCs	Cardiomyocytes	*Cao et al.*, 2011
8	Matrix sandwich + sequential treatment with Activin A, BMP4, & bFGF	Foreskin fibroblast derived hiPSCs	Cardiomyocytes	*Zhang J et al.*, 2012
9	Activin + (SB431542+ Noggin) + (EGF + FGF10 + KGF + Wnt3a)	Fibroblast derived hiPSC	Alveolar type I and II cells	*Ghaedi M et al.*, 2013
10	Wnt pathway activator, CHIR99021, and either AM580 or TTNPB	hiPSCs/hESCs	Kidney lineage cells	*Araoka et al.*, 2014
11	Retinoic acid and BMP4 treatment and culture on Collagen-I & IV dishes	hiPSC	keratinocytes	*Kogut et al.*, 2014
12	High (60%) Oxygen culture conditions	Mouse ESCs and hiPSCs	Endocrine progenitors (Insulin producing)	*Hakim et al.*, 2014

*BMP*4: *Bone morphogenetic factor*-4; *FGF*2: *Fibroblast growth factor*-2; *GSK*: *Glycogen synthase kinase*; *HGF*: *hepatocyte growth factor*; *hiPSCs*: *human induced pluripotent stem cells*; *hESCs*: *human embryonic stem cells*; *WNT*3*a*: *Wingless-Type MMTV Integration Site Family, Member* 3*A*.

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stroke which contribute enormous burden to the health system. There is a general perception that hESCs hold the solution for various reproductive health problems not involving gametes such as endometrial damage, erectile dysfunction and vaginal atrophy. However, no conclusive results have been obtained which back these claims [45].

4. Impediments in ESCs Use in Clinical Settings

Procurement of human embryos exclusively for the purpose ESC isolation has always remained morally and ethically contentious. There has been a long-standing heated argument amongst the researchers as well as the general public on the religious, moral and cultural aspects of the issue [46]. Adding more to the controversy to this debate is the issue of abortion/disposal of human embryos subsequent to their use in the IVF clinics. Furthermore, extended storage of hESCs may cause chromosomal abnormalities such as trisomy 12 and 17 and ge-

netic amplification at 20q11.21 which is associated with oncogenic transformations [25] [27] [47]. These abnormalities are generally associated with the genes determinant of pluripotency, cell proliferation, and anti-apoptosis, which is remarkably similar to the genetic changes observed in many human tumors [47]. Besides ethical and moral issues, the "non-self" nature of ESCs appends to the poor immunological acceptance of their derivative tissue by the recipient despite enjoying immunopriviledged status in their undifferentiated state. Hence, an adjunct immunosuppression therapy is essential to augment their post engraftment acceptance. The immunopriviledged status of undifferentiated hESCs has been attributed to low level expression of MHC-I and absence of MHC-II and co-stimulatory molecules CD80 and CD89. Upon differentiation, the derivative tissue expresses MHC-I thus rendering the derivative tissue immunogenic [48] [49]. There are reports however, that the cells derived from ESCs have low immunogenicity and even possess anti-inflammatory and immunosuppressive properties [50] [51].

Another concern that has seriously hampered the progress of hESCs to routine clinical application is their robust teratogenic potential [52]. Despite ample evidence from published data, the underlying reason and mechanism of hESC's teratogenic potential is largely undetermined. Given that hESCs share many similarities with the embryonic carcinoma cells (ECCs), hence tumorgenicity is one of the shared characteristic of hESCs with ECCs [53]. Moreover, teratogenicity being inherent characteristic of pluripotent stem cells; it is also associated with adaptation of the cells to the environment thus activating the oncogenic networks in the cells at molecular levels [53]. Attempts are underway to discriminate between partially transformed ESCs and normal ESCs as a strategy to curtail their tumorgenicity.

5. ESCs and the NIH Policies

Unlike the concerns pertaining to immunological acceptance and tumorigenic potential of ESC that warrant pure scientific effort for resolution, divide in the scientific and social circles regarding ethical/moral issues seem deeper. Whereas the Bush administration's policy about hESCs to ban federal funding for newly created hESCs lines dampened the hope in the scientific community involved in stem cell research, the Executive Order 13505 issued by President Obama in 2009 has been quite encouraging [54]. According to the new guidelines, embryos could only be obtained after proper informed consent from the donor without payments, cash or rewards. Moreover, the new NIH guidelines allow the use of embryos created using IVF for clinical use but are no longer needed for that purpose, provided that the donor has agreed to do so [55]. The Guidelines also apply some restrictions on the donors. Firstly, the donor is not allowed to choose the recipient of their cellular transplants including themselves. Secondly, the donor receives information that the research is not intended to provide direct medical benefit to the donor. Thirdly, in case that the results of research using the derived hESCs end up with commercial potential, the donor will not receive financial or any other benefits from any such commercial development. It is significant to note that the guidelines do not disqualify a donor from benefitting from the medical outcomes of stem cell research and treatments that may be developed in the future.

6. Induced Pluripotent Stem Cells

iPSCs are surrogate ESCs as they share common characteristics such as the expression of pluripotency markers, self-renewal, differentiation capacity to form cells from all the three germ layers, unlimited proliferative potential and teratogenicity post engraftment [56]. The acceptance of iPSCs as an alternative to ESCs has grown over the years as it surmounts the obstacles posed by the use of ESCs. Firstly, iPSCs generation does not involve technically more tedious methodologies such as procurement of oocytes and somatic nuclear transfer [57]. Secondly, unlike ESCs, there are no bioethical issues surrounding the iPSCs as a continuous source of autologous and disease specific pluripotent stem cells. Despite close similarities, iPSCs have distinct features in comparison with their ESC counterparts including maintenance of epigenetic memory of the mother cells from which they have been derived [58] [59]. Similarly, in contradiction to the general perception that iPSCs derived from autologous source would be immunologically more acceptable, recent studies have probed the immunological acceptability of iPSCs derived from autologous donor cells and warrant caution before use in the clinical settings [60]. During the course of a study to ascertain the process of pluripotency induction, a set of more than 24 candidate transcription factors relevant to pluripotency in ESCs was tested to determine the necessary factors for the development of iPSCs. Many members of the set of the tested genes showed oncogenic potential when used on animal samples. Subsequent to a series of experiments, the authors were able to shortlist the quartet of essen-

tial transcriptional factors *i.e.*, *Oct*3/4, *c-Myc*, *Sox*2 and *Klf*4, to successfully develop iPSCs [61]. Bisulfite genomic sequencing revealed that the promoters of both Fbx15 and Nanog were demethylated while the promoter of Oct3/4 remained methylated in the transformed cells. Although the efficiency of transformation was very low, the results were significant in terms of transformation of the field of stem cell therapy from being stagnant to vibrant. It wasn't until 2007 that human iPSCs were reported in one study each published by Thomson *et al.* at University of Wisconsin-Madison and Yamanaka *et al.* at Kyoto University [9] [61]. Whereas the latter study generated human iPSCs from adult dermal fibroblasts with the classical quartet of transcription factors *Oct*4, *Sox*2, *Klf*4 and *c-Myc*, the former used a combination of *Oct*4, *Sox*2, *Nanog* and *Lin*28 for successful induction of pluripotency. The iPSCs thus generated in both the studies were similar to ESCs in pluripotency and differentiation characteristics.

7. Advances in Reprogramming Strategies to Generate iPSCs

Due to incomplete understanding of the underlying molecular mechanisms that lead to cellular transformation from terminally differentiated status to pluripotency, the protocols of reprogramming are less than optimal in efficiency. Three years after successful reprogramming of mouse fibroblasts using classical quartet of transcription factors, two different models, elite model and stochastic model, were put forth to explain the reprogramming process [62]. According to the elite model, not all somatic cells were reprogrammable whereas the stochastic model predicted that all somatic cell types might be reprogrammed with the involvement of epigenetic alterations enroute to pluripotency [63]. The stochastic model was supported by many independent studies showing successful reprogramming of different cell types including skin fibroblast [61], dental pulp [64], peripheral blood cells [65], T-cells [66], bone marrow cells [67], skeletal myblasts [68] and many others. On the contrary, the elite model relies on a recently published study that the presence of multilineage-differentiating stress enduring (Muse) cells is predominantly responsible for iPSC generation from human fibroblast [69]. An interesting feature of Muse cells is that these cells, either derived from bone marrow mesenchymal stem cells or human fibroblasts, express CD40, CD90 and CD105 markers of mesenchymal lineage besides concomitant expression of pluripotency marker SSEA3 (stage specific embryonic antigen 3). Although much controversy shrouds the exactness of a model explaining the mechanism of somatic cell reprogramming, it is generally accepted that reversal of differentiated somatic cells to pluripotency is a step-wise transition rather than spontaneous/abrupt change of status [70]. The earlier deterministic phase involves mesenchymal to endothelial transition (MET) followed by stochastic phase with random probability of distribution. The stochastic phase is typified by specific microRNA expression profile, histone modifications and changes in DNA methylation status [70]. Nevertheless, the stochastic model does not fully appreciate the role of microRNA (miRNA) in MET. The maturation phase transits into stabilization of stem cell circuity activation once the cascade of these specific changes has occurred. An interesting study involving single cell reprogramming was carried out to study the molecular events that precede the transformation of a somatic cell to pluripotency [71]. Recent data vividly support a substantial role of epigenetic modifications during the cascade of reprogramming events in many types of somatic cells. A detailed description of the role of epigenetics has been discussed under "Epigenetics of iPSCs".

The protocols for direct reprogramming of somatic cells to pluripotency by ectopic expression of exogenous transcription factors have evolved significantly over the years. The modified protocols range from replacement of some members of the classical quartet to reduce the number of transcription factors and the delivery strategy from viral to non-viral vectors and even DNA-free methods based on small molecule treatment and protein transduction methods using cell penetrating peptide moieties. Protocols have also been developed by combinatorial approach based on mix of different strategies. The main motive behind refining the reprogramming protocol is three-fold: to enhance the reprogramming efficiency, eliminate the use of oncogenic factors from the classical quartet, and to make the derivative cells safer for clinical applications.

The earlier studies for refining the protocol were envisioned to understand the role of each one of the four transcription factors of reprograming. It was considered that the presence of cMyc contributed towards immortality and active status of chromatin, Oct3/4 changed the fate of the cell from tumour cells to ESC-like cells, Klf4 suppressed senescence while Sox2 was responsible for pluripotency of the derivative iPSCs [72]. The collective effect of ectopic expression of transcription factors altered the DNA methylation status, gene expression profile and chromatin status in the newly transformed cells similar to the ESCs [73]. Following the success of reprogramming protocols with 4 factors, plethora of publications ascertained the feasibility of replacingor re-

ducing the number of transcription factors required for successful reprogramming of somatic cells [10] [66] [74] [75]. Ectopic expression of as little as single factor *i.e.*, Oct4, either alone or in combination with small molecules has been attempted for successful reprogramming of somatic cells [76]-[79]. These studies also highlight the paramount significance of Oct4 and its indispensable part for success of any reprogramming protocol. The usage of small molecules to enhance the reprogramming efficiency is increasingly becoming popular due to their well-defined chemical nature, permeability to cross the cell membrane, impacting the chromatin status and various signalling pathways and allowance to adjust the concentration required to achieve the desired consequence [80]. Additionally, versatility of reprogramming strategy of somatic cells has been tested in cells from different species including mouse [8] [14], rat [81] [82], monkey [83], porcine [84] [85], and humans [8] [9]. As a part of the strategy to enhance the efficiency of reprogramming, cells from different tissue sources have been successfully employed [67] [68] [86]-[89]. This was based on the understanding that the inherent expression of one or more pluripotency factors may reduce their dependency on exogenous factors thus making these cells labile to undergo transformation to pluripotent status. For example, neuronal progenitor cells which endogenously express higher level of Sox2 were successfully reprogrammed to pluripotency by transduction of a combination of exogenous Oct3/4, Klf4 and cMyc [90].

One of the important aspects of reprogramming strategy under intense scrutiny has been the vectors for genetic manipulation of cells with pluripotency transcription factor genes. Given their marked ability and efficiency of transduction in a variety of cells with ease, the use of viral-vectors is fairly popular strategy [91]. Specifically, different viral vectors, *i.e.*, retrovirus [61], and lentivirus [9] have been successfully used for manipulation of the donor cells. The downside of viral vectors is their tendency to randomly integrate the transgenes into the host genome besides being difficult to dislodge once reprogramming has been accomplished [92]. Two non-integrating viruses, adenovirus and Sendai virus have gained popularity in the field of iPSCs reprogramming. Nevertheless, the increased rate of tetraploid cells presence after the use of adenovirus remains a big disadvantage [62]. On the other hand, Sendai virus provides a superior option because of its largely non-integrating potential, safety and overall comparatively less disadvantages [93]. Again, it remains a formidable challenge to remove the replicating virus from the reprogrammed cells with the use of Sendai virus. In order to replace viral vectors, a variety of non-viral methods have been designed and applied successfully [16]-[18]. Episomal plasmids are used for reprogramming because they are easily introduced into and removed from target cells [94]. The downside of this method is the low transfection efficiency which requires multiple rounds of transfection. Alternatively, inefficiency of non-viral methods of reprogramming is being compensated by combinatorial approach wherein the donor cells are treated with small molecules that work by activation of endogenous pluripotency determining transcription factors, in conjunction with non-viral genetic manipulation [95].

As an alternative to ectopic transgene expression of pluripotency transcription factors, protocols for manipulation of microRNA (miRNA) profile for one or more specific miRNAs with role in pluripotency determining signalling pathways have been reported. miRNAs are short non-coding segments of RNA which regulate gene expression during various cellular processes including pluripotency and differentiation of stem cells [96]. Many recent studies have suggested the role of specific miRNAs in ESCs associated with pluripotency genes [97] [98]. Similar to the core set of transcription factors relevant to the differentiation status of ESCs, there is also a core set miRNAs associated with the differentiation status of ESCs including miRNA 302-367 and miRNA 290-295. Interestingly, pluripotency determining transcription factors and the core set of ESC specific miRNA are intricately related to each other in terms of their functionality [99]. The premier usage of miRNA in reprogramming process is intended to enhance the rate and efficiency of iPSCs generation. Different miRNAs *i.e.*, 200c, 302/367 cluster and 369s family have been effectively used for reprogramming of somatic cells but with low efficiency [100] [101]. Of this core set of miRNAs, over expression of micro-302 alone may drive the process of reprogramming of somatic cells [101]. An interesting aspect of miRNAs research in relation to reprogramming is maintenance of the epigenetic memory of the parent somatic cells [102].

8. Epigenetics of iPSCs

As discussed earlier, ESCs derived from the inner cell mass and iPSCs reprogrammed from somatic cells *via* transgenic expression of pluripotency determining transcription factors possess similar differentiation capacity. On molecular level, they significantly share gene expression as well as miRNA profile besides DNA methylation status. Nonetheless, both cell types exhibit differential set of genetic and epigenetic characteristics derived

from their respective parent cells [103]. It is generally considered that the epigenome and the transcriptome of the parent cells that remain distant from the molecular events encompassing the reprogramming process are carried forward to the reprogrammed cells as part of the residual memory [104]. Indeed, pluripotent status and the subsequent differentiation characteristics of the pluripotent cells are associated with their epigenetic memory [105]. For example, immunogenicity of iPSCs is a continuation of the immunogenicity of their parent somatic cells as the immunogenicity-determining epigenome is sustained during the process of reprogramming [106]. Other studies have also highlighted that the derivative iPSCs carry forward the epigenetic memory of their respective parent cells thus defining it as arguably the key to control the efficiency of reprogramming process [103].

In terms of molecular signalling, epigenome influences the gene expression profile in a cell *via* enzyme-catalysed reactions without altering the nucleotide sequence of the genome. In the context of iPSC, polycomb group of proteins (PcG) is a particularly important determinant of epigenetic inheritance by chromatin remodelling such that transcriptional repression ensues [107]. PcG is also implicated in the maintenance of undifferentiated self-renewal of stem cells, efficiency of reprogramming and molecular pathogenesis of cancer stem cells [103] [108]. At least two PcG complexes PRC1 (plycomb repressive complex-1) and PRC2 (plycomb repressive complex-2), either alone or synergistically, repress their target genes at the nucleosome level of the chromatin structure. The EZH1/2 subunit of the PRC2 is a histone methyltransferase that trimethylates the target genes on tyrosine 27 of histone 3 (H3K27). The trimethylated H3K27 (H3K27me3) is recognized by a subunit of the PRC1 called chromobix. Their complex leads to the recruitment of yet another subunit of the PRC1, RNF2/RING1 ubiquitin ligase to attach a ubiquitin molecule to H2AK119 residue. These two epigenetic modifications make the chromatin three-dimensionally inaccessible to the transcriptional machinery. Pluripotency is preserved by maintaining a balance between transcription of pluripotency-related and lineage-specific genes with significant participation of PcG proteins to repress cell differentiation [103]. Notably however, the PcG proteins are unrelated to the pluripotency level in stem cells as they donot influence the expression levels of pluripotent markers, such as Oct4 and Nanog [108] [109].

9. Pre-Clinical and Clinical Studies with Pluripotent Stem Cells and Potential Applications

Both ESCs and iPSCs have been extensively characterized in experimental animal models for assessment of their therapeutic potential, disease modelling, and drug development (**Table 5** & **Table 6**). Mostly these studies provide ample proof of the concept that pluripotent stem cells, irrespective of their origin, can adopt functionally competent cell types belonging to all the three germ layers post-engraftment in experimental animal models. All these data are unambiguous in depicting the reparative potential of pluripotent stem cells. During two independent studies, mouse skeletal myoblast derived iPSCs as well as bone marrow derived iPSCs and their derivative cardiomyocytes effectively attenuated infarct size expansion in murine model of acute myocardial infarction [67] [68]. Fluorescence immunostaining for myogenic makers showed that the transplanted cells underwent myogenic differentiation at the site of the graft. Besides, blood vessel density was also increased in the cell transplanted hearts as compared to the control groups of animals. Nevertheless, one of the major findings in both the studies was that nearly 35% and 21% of the iPSCs transplanted animals developed cardiac tumours. These data raised serious safety concerns and a note of caution regarding clinical utility of iPSCs. However, use of cardiac progenitors instead of iPSCs during transplantation studies alleviated the teratogenic concerns. Despite these encouraging data with progenitor cells, both pluripotent stem cells and their progenitors warrant further investigation for safety and observable differences in transcriptome before regular clinical applications in the humans. It's imperative to appreciate that some of the therapeutic applications of iPSCs are being investigated in clinical trials; such as its application in treating macular degeneration [110].

The other two areas in which iPSC have been exploited are disease modelling and drug testing [111]. The strategy involves reprograming of patient-specific cells to pluripotency followed by their differentiation into the cell type that is involved in the disease process (**Figure 2**). These derivative cells are used as a model to decipher the evolution and molecular pathogenesis of the disease in question besides their use as a platform to test novel molecules with potential for drug development. Some examples of clinical studies currently underway are intended for developing disease specific iPSCs for use *in vitro* as disease models (**Table 7**). Spinal muscular dystrophy in this regard is a typical example of the most well-studied neurodegenerative genetic disorders which

Table 5. Experimental and clinical studies using ESCs.

	Experimental animal model	Cell Type/delivery	Important findings	Teratogenicity	References
1	Rat model of traumatic spinal injury. Cell transplantation at 9 days after injury.	Mouse ESC derived neural cells/ Direct transplantation at the injured site.	Survival of the transplanted cells until 5 weeks of observation and differentiation into astrocytes, oligodendrocytes and neurons.	Not reported	*McDonald et al*., 1999.
2	SCID mice	hESCs derived PECAM+ cells. PLGA/PLLA patch or scaffold based s.c implantation.	New micro vessels with human endothelial cells lining observed on day 7 and 14 after engraftment.	Not reported	*Levenberg et al*., 2002
3	(MPTP-treated) monkeys, a primate model for Parkinson's disease	Dopaminergic neurons from monkey ESC derived neurospheres treated with FGF20 and FGF2 to for differentiation. Direct injection into bilateral putamen.	Functional engraftment of dopaminergic neurons to alleviate MPTP-induced neurological symptoms	Not reported	*Takagi et al.*, 2005
4	Athymic male rat heart model of I/R injury.	hESC derived cardiomyocytes and treated with pro-survival cocktail. Cell transplantation 4 days after I/R injury by direct intramyocardial injection.	Engraftment and survival and integration of the transplanted cells. Significant attenuation of LV remodeling and preservation of cardiac function	Not reported	*Laflamme et al*., 2005
5	Mouse model of myogenesis using cardiotoxin injection alone or with 25 Gy X-radiation.	hESCs derived myogenic precursors/IM injection.	Myogenic differentiation of xenotransplanted precursors.	No evidence of teratogenicity until 128 days of observation.	*Zheng et al.*, 2006
6	Rat model of myocardial infarction by coronary artery ligation with immunosuppression.	Direct intra-myocardial injection of hESC derived CMs and Non-CMs.	Integration of hESCs derived CMs with host CMs via gap junctions, attenuated LV-remodeling and improved LVFS.	No teratomas observed in hESC derived CMs.	*Caspi et al.*, 2007
7	Porcine model of retinal stem cell transplantation prepared by treatment of iodoacetic acid to eliminate photoreceptors	Swine iPSCs derived Rho + rod photoreceptors transplanted in to sub-retinal space of pig eye.	Successful engraftment and integration of iPSCs-derived Rho + photoreceptors into damaged neural retina of pig.	Not reported	*Zhou et al.*, 2011
8	SCID/beige mice (deficient in B and T lymphocytes and NK cells) with alloxan induced diabetes. Subcutaneous implantation model.	hESCs and their derivative pancreatic islet progenitor cells loaded into bilaminar theracytes. Subcutaneous implantation of the loaded theracytes,	Cells remain fully encapsulated. The derivative pancreatic islet cells secreted sufficient insulin to alleviate diabetes at 20 weeks after implantation.	Not reported	*Kirk et al.*, 2014
9	Macaque myocardial model of ischemia followed by reperfusion.	hESCs derived CM intramyocardial delivery	Extensive re-muscularization of myocardium and electro-mechanical junctions between donor and host myocytes. Non-fatal arrhythmias.	Not observed until 84 days of observation.	*Chong et al.*, 2014
10	Patients with Stargardt's macular dystrophy and dry age-related macular degeneration	hESC	retinal pigmented epithelium (RPE)	absent in an observation period of 4 months	*Schwartz*, 2012

CMs: *Cardiomyocytes*; *hESCs*: *Human embryonic stem cells*; *IM*: *Intramuscular*; *I/R*: *Ischemia-reperfusion*; *LV*: *Left ventricle*; *LVFS*: *Left ventricular fractional shortening*; *MPTP*: 1-*methyl*-4-*phenyl*-1,2,3,6-*tetrahydropyridine*; *s.c.*: *Subcutaneous*; *SCID*: *severe combined immunodeficiency*;

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Table 6. Experimental animal studies using iPSCs and their derivative progenitor cells.

#	Experimental animal model	Cell type	Route of delivery	Important findings	Teratogenicity	Reference
1	Mice (adult wild-type mice)	hiPSC-derived fibroblasts	Direct injection into the sub-retinal space	Photoreceptors (retinal cells)	No teratomas were found following transplantation	*Lamba et al.*, 2010
2	Mouse model of contusive spinal cord injury	Safe iPSCs derived neurospheres	Direct injection into lesion epicenter	Neuronal differentiation	Safe iPSCs derived cells were non-teratogenic	*Tsuji et al.*, 2010
3	Hind-limb ischemia model of SCID mice by femoral artery ligation	hiPSC derived endothelial cells	Direct intramuscular injection	Increased capillary density and regional perfusion	No teratomas reported	*Jalil et al.*, 2011
4	Mouse model of acute coronary artery ligation	Skeletal myoblast derived iPSCs	Direct intra-myocardial injection	Angiomyogenesis with preserved heart function	35% animals with iPSCs and no teratomas with progenitor cells	*Ahmed et al.*, 2012
5	Porcine model of myocardial infarction	hiPSCs	Catheter based I/M	Endothelial differentiation and neoangiogenesis	None until 12 - 15 weeks after transplantation	*Templin et al.*, 2012
6	Ischemic cardiomyopathy with ameroid ring	hiPSCs derived cardiomyocytes	Patch based delivery	Cardiomyogenesis with preserved LV remodeling and heart function	No teratogenicity during the course of studies	*Kawamura et al.*, 2012
7	Mouse model of acute coronary artery ligation	Mouse MSC-derived iPSCs or their derived cardiac progenitors.	Direct intra-myocardial injection	Angiomyogenesis with preserved heart function	21% animals developed teratomas with iPSCs and no teratomas with progenitor cells	*Buccini et al.*, 2012
8	Mice (7- to 8-week-old female nude mice and immunodeficient mouse strains (nude, SCID, NOD-SCID and NOG)	Dermal fibroblast derived hiPSC	Direct subcutaneous injection	Retinal pigment epithelium	Negative after 15 months	*Kanemura H et al.*, 2014

hiPSCs: *human induced pluripotent stem cells*; *iPSCs*: *induced pluripotent stem cells*; *I/M*: *intramyocardial injection*; *MSC*: *mesenchymal stem cells*; *SCID*: *severe combined immunodeficiency*. 1. Lamba D, McUsic A, Hirata R, *et al*. Generation, Purification and Transplantation of Photoreceptors Derived from Human Induced Pluripotent Stem Cells. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2808350/. 2. Tsuji O, Miura K, Okada Y, *et al*. Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proc Nat AcadSci USA. 2010; 107: 12704-9. 3. Ahmed RP, Haider HK, Buccini S, Li L, Jiang S, Ashraf M. Reprogramming of skeletal myoblasts for induction of pluripotency for tumor-free cardiomyogenesis in the infarcted heart. Cir Res. 2011; 109: 60-70. 4. Jalil RA, Huang NF, Jame S, Lee J, Nguyen HN, Byers B, De A, Okogbaa J, Rollins M, Reijo-Pera R, Gambhir SJ, Cooke JP. Endothelial cells derived from human ipscs increase capillary density and improve perfusion in a mouse model of peripheral arterial disease. ArteriosclThrombVasc Biol. 2011; 31: e72-79. 5. Templin C, Zweigerdt R, Schwanke K, Olmer R, Ghadri JR, Maximilian Y. Emmert MY, Müller E, Küest SM, Cohrs S, Schibli R, Kronen P, Monika Hilbe M, Reinisch A, Strunk D, Haverich A, Hoerstrup S, Lüscher TF, Kaufmann PA, Landmesser U, Martin U. Transplantation and tracking of human-induced pluripotent stem cells in a pig model of myocardial infarction: Assessment of cell survival, engraftment, and distribution by hybrid single photon emission computed tomography/computed tomography of sodium iodide symporter transgene expression. Circulation. 2012; 126: 430-439. 6. Kawamura M, Miyagawa S, Miki K, Saito A, Fukushima S, Higuchi T, Kawamura T, Kuratani T, Daimon T, Shimizu T, Okano T, Sawa Y. Feasibility, Safety, and Therapeutic Efficacy of Human Induced Pluripotent Stem Cell-Derived Cardiomyocyte Sheets in a Porcine Ischemic Cardiomyopathy Model. Circulation. 2012; 126 [suppl 1]: S29-S37. 7. Stephanie Buccini S, HaiderHKh, Ahmed RPH, Jiang S. Cardiac progenitors derived from reprogrammed mesenchymal stem cells contribute to angiomyogenic repair of the infarcted heart.Basic Res Cardiol. 2012; 107: 301. 8. Kanemura H, Nishishita N, Shikamura M, *et al*. Tumorigenicity studies of induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium (RPE) for the treatment of age-related macular degeneration. Retrieved from http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0085336#s4.

lead to infant mortality [112]. Prior to the use of patient specific iPSC derived motor neurones, patient derived fibroblasts were mainly used to study the disease process. Given that motor neurones have specific features not shared by fibroblasts, the use of iPSC derived disease specific motor neurones provide superior and more relevant *ex vivo* disease model [113]. Since the publication of these data, similar studies have also been reported for both haematological and non-haematological disorders [114]. The use of iPSCs for generation of disease specific models carries several advantages. Firstly, in most cases, the availability of the desired cell types from the patient required for use as a model remains problematic. iPSCs have solved this problem by providing an unlimited

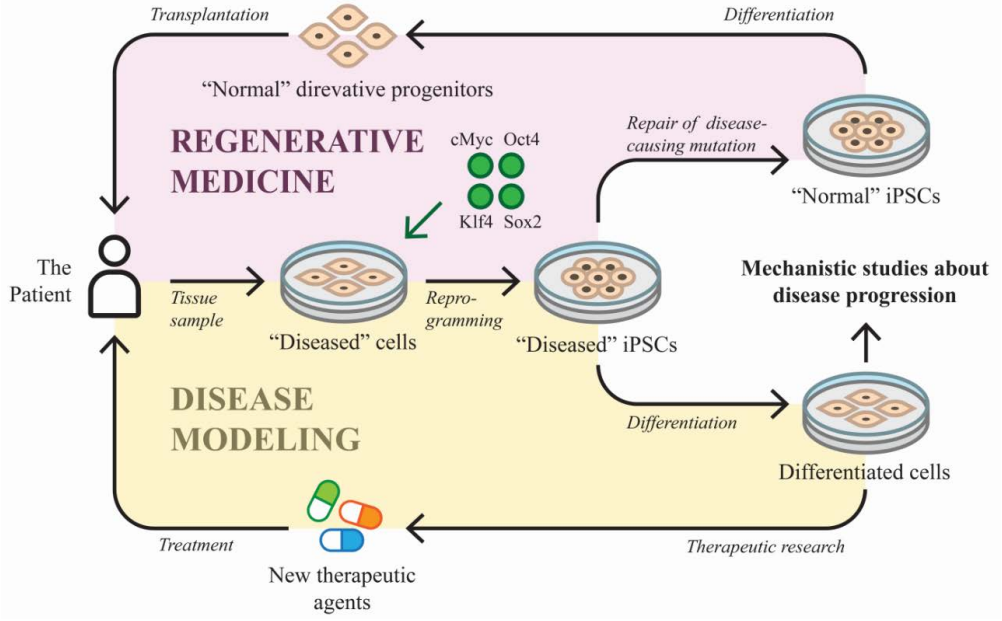

Figure 2. Potential applications of iPSCs.

Table 7. Clinical studies involving iPSCs generated from the somatic cells donated by the patients with different pathological disorders. The prime objective of these studies is to study the disease mechanism and drug research rather than regenerative or reparative use in the human subjects.

Clinical Trials.gov Identifier	Location and Investigator	Title of the study	Purpose	Start Date	Completion date	Cells involved
NCT01943383	Julie A Johnson, Pharm D. University of Florida, USA	Pharmacogenomic evaluation of antihypertensive responses in iPSC study (PEAR-iPSC)	To study the variation in responsiveness of antihypertensive drug therapy in different individuals.	August 2013	October 2016	iPSCs
NCT02246491	Sonia Franco, MD. Sidney Kimmel Comprehensive Cancer Center, John Hopkins, USA	iPSC-based approaches for modelling and treating Ataxia-Telangiectasia	To understand the disease mechanism of Ataxia-Telangiectasia (A-T) using patient fibroblast derived iPSC *in vitro*.	October 2014	October 2017	iPSCs
NCT02193724	Rachel C. Brennan, MD St. Jude Children's Research Hospital (University of Wisconsin, Madison, USA: collaborator)	Feasibility, validation and differentiation of iPSC produced from patients with heritable retinoblastoma	To establish the feasibility of producing iPSCs from retinoblastoma patients with germline RB1 mutations, validate the derivative cells and differentiate them into retina as a model of the initiation of retinoblastoma in the developing retina.	October 2014	April 2017	iPSCs
NCT02056613	David F Stroncek, M.DNational Institutes of Health Clinical Centre, USA	Blood collection from healthy volunteers and patients for the production of clinical grade iPSC products.	To develop new methods to make iPSCs; to identify better ways to collect, produce, and grow them; and to make an iPSC bank.	January 2014	October 2018	iPSCs
NCT00874783	Benjamin E Reubinoff, MD., PhD. Hadassah Medical Organization, USA	Derivation of iPSCs from somatic cells donated by patients with neurological diseases for the study of the pathogenesis of the disorders and development of novel therapies.	The major goal of the project is to develop human iPSCs from cell cultures from skin biopsies or the patient's hair for modelling diseases and drug discovery and for developing the technology that may eventually allow the use of iPS cells for future transplantation therapy.	April, 2009	December, 2018	iPSCs

Source: https://clinicaltrials.gov/ct2/results?term=Induced+pluripotent+stem+cells&Search=Search.

continuous source of the cells in question. Secondly, iPSC derivation of cells for a disease model allows temporal observation of the course of disease process thus enhancing the complete understanding of the molecular pathogenesis of the disease in question. Despite these advantages, this "disease in a petri dish approach" also has many limitations. Although the approach ensures continuum of disease specific cells for study, it necessitates disease specific iPSC generation from multiple patients to cover full span of the possible phenotypic variations from differing levels of the severity of the disease. Additionally, differentiation of disease specific iPSCs to obtain the disease specific cells necessitates optimization such that the derivative cells more closely resemble the cells present in the patient during the natural course of the disease process. Moreover, such applications may be restricted to monogenetic diseases only and would require in-depth research for more complex multifactorial diseases. Despite these limitations, iPSC derived disease specific cells are significant addition to the *ex vivo* models for pharmacological studies and toxicity assessment of novel compound libraries for high throughput screening during drug development [115]-[117].

10. Conclusion

As discussed earlier, iPSCs have multiple potential applications that can be classified into three main areas of interest: therapeutic applications, disease modelling, and drug testing. Despite encouraging results, the potential application of iPSC in tissue transplantation and repairing disease-causing mutations by homologous recombination has yet to show its effectiveness in routine clinical practice. While iPSCs have overcome the issues encountered by their counterpart ESCs and other stem cell types including immune tolerance and ethical controversies, the safety of its use in human remains a major impediment. Some of the safety concerns imply their potential tumorigenicity and the observable differences in the transcriptome of iPSCs and ESCs as discussed earlier. It is worthwhile to appreciate that iPSCs have entered into clinical trials for reparation and restitution of diseased tissues as the prime objective such as their application for the treatment of macular degeneration. Although the trial has been suspended on account of failure of the cells to pass genetic validation for the second patient enrolled in the study, this is a big leap that has created new hope for the use of pluripotent stem cells.

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List of Abbreviations

c-Myc	c-Myelocytomatosis
EBs	Ebryoid Bodies
ECM	Extracellular Matrix
hESCs	Human Embryonic Stem Cells
ICM	Inner Cell Mass
iPSCs	Induced Pluripotent Stem Cells
Klf4	Krupple Like Factor-4
LIF	Leukemia Inhibitory Factor
MHC	Major Histocompatibility Complex
mESCs	Mouse Embryonic Stem Cells
NIH	National Institute of Health
Oct4	Octamer-4
Sox2	Sry Box Containing Gene 2