Pluripotent stem cells in regenerative medicine: challenges and recent progress

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Abstract

After years of incremental progress, several recent studies have succeeded in deriving disease-relevant cell types from human pluripotent stem cell (hPSC) sources. The prospect of an unlimited cell source, combined with promising preclinical data, indicates that hPSC technology may be on the verge of clinical translation. In this Review, we discuss recent progress in directed differentiation, some of the new technologies that have facilitated the success of hPSC therapies and the remaining hurdles on the road towards developing hPSC-based cell therapies.

Access to unlimited numbers of specific cell types on demand has been a long-standing goal in regenerative medicine. With the availability of human pluripotent stem cells (hPSCs) and greatly improved protocols for their directed differentiation, this prospect could become a reality for several disease-relevant cell types. Recent advances in the stem cell field indicate that the 'holy grail' of directed differentiation (that is, the generation of unlimited numbers of authentic and genetically matched cell types for cell therapy) could indeed translate into effective therapies for currently intractable disorders[1–4], although new challenges are likely to emerge on the road towards such translation in humans.

In parallel to the improvement in directed differentiation, novel technologies have been developed to assess lineage, fate and function of stem cell-derived cell types both *in vitro* and *in vivo*. Here, we review some of the recent breakthroughs in directed differentiation and discuss their implications for cell therapy. The ability to access patient-specific cells at scale and on demand are also crucial for human disease modelling and hPSC-based drug discovery; these applications are not discussed here but have been the subject of a recent review[5].

In this Review, we present recent breakthroughs in deriving therapeutically relevant cell types from hPSC sources, discuss some of the tools that made such progress possible and highlight crucial next steps and challenges on the road towards clinical translation.

Deriving neural cell types

Neural cell types were among the first lineages to be reliably obtained from hPSC sources[6,7]. Arguably, most progress has been made in the area of neuronal lineage specification, which is highly dependent on mimicking *in vitro* the early patterning signals that impart axial coordinates during neural development. Both small-molecule-based and morphogen-based approaches have been developed to derive specific neuronal subtypes from pluripotent stem cells. However, the replacement of nerve cells in traumatic or degenerative disorders of the central nervous system (CNS) remains a daunting task. Recent strategies for *in vivo* cell-fate conversion are still at early stages of development but could potentially advance as an alternative approach that bypasses the need for cell transplantation (reviewed in REF. 8).

Over the years, the field of directed differentiation has used three main strategies to specify neural lineages from hPSCs. These strategies are embryoid body formation, co-culture on neural-inducing feeders and direct neural induction. Early protocols for embryoid body formation were based on triggering differentiation of human embryonic stem cells (hESCs) followed by selection in serum-free media to enrich for neural lineages[6]. The development of serum-free embryoid body cultures enabled the direct induction of neural lineages from hPSCs, and the efficiency of serum-free embryoid body formation could be greatly improved in the presence of the Rho-associated protein kinase (ROCK) inhibitor compound Y-27632 (REF. 9) that prevents cell death of dissociated hPSCs. Stromal feeder-based cultures have also been widely used for generating neuroepithelial cells and specific neural populations, including midbrain dopamine neuron-like cells from hPSCs[10]. Although the mechanism of neural induction (that is, stromal-derived inducing activity) remains unclear and the use of feeders would greatly complicate translational use, this approach has remained in use because of the robust induction efficiencies and the ability to combine it with other neural inducing strategies.

Direct induction protocols do not require embryoid body formation or co-culture for neural induction. Early attempts at direct conversion were based on the simple switch of hESC cultures to serum-free culture conditions followed by mechanical isolation of spontaneously appearing neural rosette cultures[7]. However, the use of defined neural inducers, such as inhibitors of transforming growth factor (TGF) and bone morphogenetic protein (BMP) signalling (that is, dual SMAD inhibition (dSMADi)), have greatly enhanced the efficiency and the speed of neural induction[11]. A particularly attractive feature of dSMADi is the synchronized differentiation process that yields a nearly uniform population of early neural cells within ten days of differentiation. The use of precise patterning strategies in combination with dSMADi results in protocols for the derivation of many CNS and peripheral nervous system (PNS) lineages from hPSCs. However, regardless of the specific neural induction strategy used, the main challenge over the past ten years has been to develop protocols that implement *in vitro* the early patterning events that are responsible for creating specific neuronal and glial cell types. Only recently have these strategies been refined to a level that is sufficient to contemplate translational applications for a subset of neural lineages. Recent progress for three relevant hPSC-derived neural lineages is discussed below (FIG. 1).

Dopamine neurons

Parkinson's disease is the second most common neurodegenerative disorder and is characterized by the progressive loss of several neural cell types in the CNS and PNS. Although the causes of Parkinson's disease remain unknown to a large extent, the specific loss of midbrain dopamine neurons in Parkinson's disease is responsible for most of the motor symptoms of the disease, and most current drugs for Parkinson's disease are aimed at restoring dopamine function. Dopamine neuron replacement has therefore been pursued as a potential therapeutic strategy for many decades. The feasibility of dopamine neuron replacement has been shown using human fetal tissue in more than 300 patients with Parkinson's disease worldwide[12]. However, despite long-term *in vivo* dopamine neuron survival and dopamine secretion, the clinical benefit of using fetal dopamine neurons was modest, and a subset of patients developed troubling side effects such as graft-induced dyskinesia[13,14]. The results of both open-label and placebo controlled studies are discussed in detail elsewhere[15]. Although the interpretation of these fetal tissue-grafting studies remains controversial, there is a general agreement that fetal tissue is not a suitable cell source for developing a clinically competitive future therapy against available pharmacological and surgical alternatives, and that stem cell-based therapy must overcome the limitations of fetal tissues to become a meaningful therapeutic option.

For nearly a decade, studies had shown the derivation of cells with midbrain dopamine neuron-like properties[10,16,17]. However, those neurons lacked a subset of features, such as expression of the DNA-binding forkhead box protein A2 (FOXA2), and did not engraft efficiently. We recently established a novel protocol for deriving neurons from hESCs that is based on a more precise developmental patterning of the cells (FIG. 1a). A key feature of this protocol is the transition of the cells through a floor plate intermediate stage instead of the neuroepithelial intermediate that is used in past attempts. The floor plate is a transient developmental structure that has only been recently implicated in midbrain dopamine neuron development. The resulting floor plate-derived dopamine neurons have genetic, biochemical and physiological features of authentic midbrain dopamine neurons, and they have been successfully tested in mouse, rat and rhesus monkey models of Parkinson's disease[1]. A key step was the activation of canonical WNT signalling using a small-molecule inhibitor of glycogen synthase kinase (GSK), which is a strategy that has been replicated in several additional studies since then[18,19]. These data emphasize the importance of fully defining the identity of hESC-derived neurons before implantation.

Striatal neurons

Huntington's disease is a currently untreatable autosomal dominant neurodegenerative disease and is characterized by abnormal movements, cognitive decline and various psychiatric problems. The genetic cause of the disease is an expansion of CAG repeats within the huntingtin gene (*HTT*)[20]. Medium spiny striatal neurons are the most severely affected cell type in patients with Huntington's disease. There is a long history of fetal tissue-grafting studies in Huntington's disease[21] that aimed to replace striatal neurons. Similarly to Parkinson's disease, decades of experience from the fetal tissue-grafting studies could pave the way for future hPSC-based strategies in the treatment of Huntington's disease. A major feature of Huntington's disease is the need to restore long-distance

connectivity from the striatum to pallidal targets, which are located in the globus pallidus. A recent study[18] presents a protocol that yields a large proportion of surviving neurons that express DARPP32 (also known as PPP1R1B, which is a marker for spiny neurons) *in vivo* (FIG. 1b). It further provides evidence of efferent connectivity and behavioural improvement in a striatal lesion model, which suggests considerable translational potential. However, the key factors that led to the improved performance remain unclear, as the new protocol did not include a novel strategy but optimized existing conditions (that is, sonic hedgehog (SHH) activation and WNT inhibition)[22]. Ongoing developmental studies[23] are geared towards further refining the derivation of DARPP32+ striatal neurons.

Glial cells

The derivation of engraftable glial cells — for example, by myelinating oligodendrocytes — presents a different set of challenges, such as protracted developmental timing. Although early studies suggested that cells with oligodendroglial properties can be rapidly derived from hESCs[24], subsequent studies argued that those early lineage cells may not be truly committed and require months of maturation for *in vivo* myelination. Nevertheless, those early glial precursors have developed into the first hESC-based product to reach clinical trials for treating spinal cord injury[25]. More committed cells from the oligodendrocyte lineage were obtained following long-term *in vitro* culture upon early exposure to retinoic acid and SHH agonists[26]. The translational potential of hPSC-derived oligodendrocyte precursors was best illustrated in a recent study using glial precursors that were derived from a long-term *in vitro* differentiation of human induced pluripotent stem cells (iPSCs), which were then grafted into the neonatal CNS of myelin-deficient mice[3] (FIG. 1c). Under those conditions the precursors showed extensive remyelination *in vivo* and significantly extended lifespan in the transplanted mice. However, the need for extensive *in vitro* differentiation before transplantation (that is, 125 days) may hamper clinical translation. Furthermore, it may be advantageous to develop culture conditions that exclusively yield oligodendrocytes, as the current grafts also contain many astrocytes. Finally, it will be important to graft these derived cells into specific locations of the adult CNS, where cell migration is likely to be more limited, and into large animal models where the scale of remyelination is more challenging. Several therapies are currently being developed using hPSC-derived oligodendroglial cells; these include treatment for rare genetic disorders, late-stage multiple sclerosis and patients with cancer who suffer from the long-term side effects of brain irradiation (reviewed in REF. 27).

Deriving non-neural cell types

The therapeutic development for many cell lineages outside the nervous system — such as hPSC-derived liver[28], endothelial cells[29] and pancreatic islets[30], as well as engraftable haematopoietic stem cells[31] — have hit major 'roadblocks'. These challenges include limited *in vivo* long-term survival and functional integration despite promising marker data *in vitro*. Here, we briefly discuss current progress in the development of hPSC-based cell therapies for cardiac repair and islet cell replacement as two examples in which sufficient progress has been made to support clinical translation in the foreseeable future despite considerable remaining challenges (FIG. 2).

Cardiac cells

The derivation of cardiomyocytes — the cells that make up the cardiac muscle — has been an important goal in hPSC research given the fact that cardiovascular disease represents the leading cause of death worldwide. There has been considerable progress in the past few years in developing defined protocols for both cardiac specification and the derivation of functional cardiomyocytes. One of the two most successful strategies that are currently being developed to generate cardiac cells at high efficiencies is a monolayer-based differentiation[32] that is based on the sequential exposure of differentiating hPSCs to activin A and BMP4. The other strategy is an embryoid body-based differentiation paradigm that follows a more complex series of defined patterning signals[33].

Both strategies are currently under preclinical development, and a recent paper has shown robust survival of hESC-derived cardiomyocytes and the ability of these cells both to couple with host cells and to suppress arrhythmias in injured hearts[4] (FIG. 2a). Ongoing studies confirm robust survival of hESC-derived cardiomyocytes but report the induction of transient arrhythmias, which disappears completely over a period of weeks to months. Immature cells transiently express features of pacemaker cells that may result in a propensity to autonomously trigger cardiac contractions. The development of methods to increase cell maturation, such as through the use of tissue engineering approaches[34], will be a major focus towards the clinical translation of this technology in humans.

Pancreatic islet cells

The development of a cell-based therapy for type I diabetes has been at the forefront of efforts in pluripotent stem cell research. Similarly to Parkinson's disease and Huntington's disease, there has been a history of fetal cell-grafting approaches and, in the case of islet cell replacement, grafting of cadaver material (for example, the Edmonton protocol). A specific problem in type I diabetes is the need to address the underlying autoimmune disorder, in addition to replacing the specific cells that are lost owing to the disease. In the past few years, excellent protocols have been developed for the induction of early endodermal and pancreatic lineages from hPSCs[35–37]. The main challenge has been the derivation of engraftable cells that are glucose responsive, that show high levels of insulin production and that do not co-express other hormones. A strategy to bypass the limited functionality of hPSC-derived, insulin-producing β-cells is the transplantation of pancreatic precursor cells that are subsequently matured *in vivo*[38] (FIG. 2b). Although *in vivo* maturation seems to facilitate the derivation of functional β-cells, the use of proliferating precursors might result in additional risks for clinical translation. Therefore, current translational efforts include the development of an encapsulation technology to address concerns about cell overgrowth. Another valuable step in the development of a clinically meaningful differentiation protocol is the use of surface markers such as CD142 to enrich for pancreatic precursor cells that have the highest capacity for *in vivo* islet cell production[30]. These strategies may enable the current protocols to move towards early-stage clinical studies. However, the development of a long-term, competitive hPSC-based islet-cell therapy for the routine treatment of type I diabetes is likely to require new technology to obtain hPSC-derived cells that are fully scalable and functional without the need for extensive *in vivo* proliferation and maturation.

Considerations for cell differentiation

Choice of cell source and developmental consideration

Pluripotent stem cells represent a particularly attractive cell source given their scalability and versatility. However, for most hPSC-derived lineages, a major challenge that remains is to harness the broad differentiation potential of these cells to generate a cell product that is suitable for clinical use. This problem is particularly acute for cell types that develop their full functionality only at later stages of development, such as haematopoietic stem cells with adult homing capacity, cardiac cells with adult-like physiology and pancreatic cells that show normal regulation in response to glucose stimulation. Current differentiation strategies for hPSCs yield differentiated cell types that most closely match embryonic or fetal stages of development. Therefore, technologies that enable the production of cell types with adult-like functional properties need to be developed in the future.

Harnessing the signalling pathways that control normal development has developed into a powerful strategy to direct hPSC fate. An obvious question is whether a better understanding of the developmental requirements will enable researchers to overcome the remaining roadblocks in hPSC differentiation and to establish protocols for any of the cell types in our body. In some instances, such as in the case of skeletal muscle precursors[39], early hPSC-based protocols were not robust enough to yield large numbers of engraftable myoblasts. However, more efficient engraftment, including functional integration into the muscle of dystrophic mice, was observed in hESC-derived cells with an inducible expression of paired box protein PAX-7 (REF. 40). It will be interesting to see whether refined patterning strategies will enable the derivation of differentiated cells, including skeletal muscle, using purely extrinsic signals[40] or whether there remains a need for either transient or stable genetic modification in the specification of certain cell lineages. Examples of new developments in directed differentiation are discussed below (FIG. 3).

Small molecules

The use of small molecules was an important tool in improving hPSC-based directed differentiation protocols (reviewed in REF. 41). Small molecules are typically less costly than recombinant protein factors and show higher potency and scalability[42]; they also enable the direct manipulation of intracellular pathways. Interestingly, off-target effects have not caused substantial problems in directed differentiation paradigms. Another surprising feature of small-molecule-based studies is that, in several cases, the inhibition rather than the activation of signalling pathways is crucial for fate specification. In the case of neural induction, the combined inhibition of BMP and Nodal–activin–TGFβ signalling (that is, dSMADi[11]) has emerged as a powerful platform for generating specific neural lineages. The key advantage of this approach is the highly synchronized and efficient nature of neural induction that enables precise temporal patterning of cells[1]. A similar approach has been adopted for the specification of endodermal derivatives, such as cells of the lung and thyroid lineages[43]. To push the limits of this approach, we have recently described a rapid induction protocol that yields hPSC-derived neurons within ten days of differentiation using a combination of five inhibitors[44]. Medically important neurons, such as pain-sensing nociceptors, were thus derived at an unprecedented speed by targeting BMP, Nodal–

activin–TGFβ, fibroblast growth factor (FGF), Notch and WNT signalling pathways. Combined small-molecule approaches may be useful for other hPSC-derived lineages, such as pancreatic precursors[35]. The ability to shorten the timing of human cell-fate specification *in vitro*, as shown for the rapid induction of human nociceptors[44], may be a useful strategy to simplify the induction of both late-born and late-maturing cell types, such as oligodendrocytes and cortical interneurons. The effect of small-molecule approaches was particularly marked for improving direct neural induction protocols. However, small molecules are widely used in various neural induction paradigms, including feeder-based[45] and embryoid body-based[46,47] neural differentiation protocols for hESCs or iPSCs[46].

Self-organization and differentiation in three dimensions

The establishment of proper three-dimensional cytoarchitecture is a crucial feature for several cell types. For example, although the first clinical trials using hPSC-derived retinal pigment epithelial (RPE) cells for treating macular degeneration were based on injecting dissociated cells[47], there are extensive efforts to generate sheets of RPE cells to ensure proper organization of these cells following transplantation into the patient's eye. The formation of three-dimensional aggregates has been a feature of several recent directed differentiation protocols, such as the derivation of pituitary cells from mouse embryonic stem cells, the generation of human or mouse optic cup-like structures and the derivation of cortical and cerebral organoid[48] cultures, as well as intestinal crypt structures, from hESCs (reviewed in REF. 49). Under those conditions, the main strategy is to enable differentiating hPSCs to reveal their self-organizing properties without the requirement for any specific extrinsic cues. Parameters that do affect the differentiation process in a self-organizing paradigm include initial cell density and overall medium composition. Some of those studies also rely on the use of specific extracellular matrix components, such as hPSC-derived cerebral organoids that require embedding early neural rosettes in a matrigel plug before the formation of organoid structures. Directing differentiation of hPSCs by manipulating extracellular matrix components has developed into a strategy for various hPSC-derived lineages, such as vascular cells[50], bone[51] and cartilage[52], and such approach is probably applicable to many other lineages as reviewed in REF. 53. However, the relative requirements for three-dimensional organization are variable. For example, a recent study using hESC-derived cardiomyocytes reported evidence for functional integration and coupling of grafted cardiomyocytes with host cells in the guinea pig heart without the requirement of cell assembly before grafting[4]. Similarly, fetal midbrain-grafting studies in models of Parkinson's disease, which is considered to be the 'gold standard' for future hPSC-grafting paradigms, did not show any benefit in grafting tissue pieces with intact cytoarchitecture compared to cell suspension grafts[54]. One example in which three-dimensional organization may have partially overcome a functional bottleneck in hPSC differentiation is the establishment of engraftable liver bud-like structures. In this example, co-culture with endothelial and mesenchymal cells resulted in structures that showed improved functionality in producing key liver enzymes[55].

Cell purification

There has been extensive progress in developing genetic reporter lines to optimize directed differentiation and to prospectively purify defined cell types for downstream applications.

Although early studies were commonly based on either small plasmid-based reporter constructs or transgenesis using bacterial artificial chromosomes (reviewed in REF. 56), more recent studies have focused on the use of reporter lines that are established by gene targeting given the increasing efficiency at which homologous recombination can be achieved in hPSCs using technologies that are based on zinc-finger nucleases, transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPRs) (reviewed in REF. 57). Such studies have been useful in defining the appropriate stage of midbrain dopamine neurons for grafting by comparing three genetically defined stages from dividing precursors to differentiated neurons[58]. Other recent examples include the isolation of homeobox protein NKX-2.1–GFP^{+} cells for subsequent derivation of highly enriched populations of cortical interneurons[59,60]. However, for translational applications it may be beneficial to avoid the genetic modification of cells. A powerful technology to facilitate the transition from a genetic-based isolation strategy to an antibody-based isolation strategy is the use of surface marker antibody screens, whereby 242–370 commercially available antibodies are screened against cells in a specific stage of differentiation. This strategy was useful for developing enrichment strategies for neural stem cells and neurons[61], as well as for various non-neural lineages such as hESC-derived cardiomyocytes[62].

Defining cell fate and function

Novel assays to characterize cell fate *in vitro*

The identification of defined cell types and stages *in vitro* is an important component of any directed differentiation strategy. Clearly, it is important to define the expression of cell type-specific markers at both the gene and protein levels. Similarly, it is vital to confirm the absence of markers that should not be expressed in a given cell type. Flow-based quantification of marker expression is particularly useful, as it circumvents the challenges that are associated with image analyses in cultures plates that contain a mixture of cell clusters and with individual cells that are difficult to quantify. However, beyond simple candidate marker expression, unbiased genome-wide analyses for transcription, such as microarray and RNA sequencing platforms, are increasingly being used as a standard in the field. There is also an increasing effort both to include global assays that define the chromatin state of differentiated hPSC progeny, as recently shown for hPSC-derived cardiac precursors[63], and to define the enhancer landscape in hPSC-derived neural crest precursors[64]. As directed differentiation of hPSCs relies on developmental signalling events that control normal embryonic development, the demonstration that hPSC-derived cultures transit through appropriate developmental intermediate stages is also important in assessing the authenticity of the final differentiated cell fate.

Assays to assess *in vivo* survival and function

Early transplantation studies were mostly limited to measuring cell survival by histology and by correlating those results with behavioural outcome. However, new imaging and functional tools aim to change the field by enabling real-time monitoring of graft survival, function and cellular integration. Advances in positron emission tomography (PET), optical imaging and magnetic resonance imaging (MRI) have steadily pushed the boundaries of

spatial resolution, especially with the use of nanoparticles, thus allowing the development of accurate spatiotemporal maps of grafts and even at a single-cell level in some cases[65]. Some of those technologies have not fully reached the clinic yet, but their preclinical development is evolving at a rapid pace[66]. Furthermore, bioluminescent imaging and magnetic labelling of grafted cells have been successfully used to track human neural, endothelial and skeletal muscle precursors[39] *in vivo*. Strategies that are currently under development for use in hPSC-derived lineages include genetic reporters that allow induced-fate mapping and *in vivo* clonal analyses, which are routinely used in mice[67]. Genetic tracking has already been used clinically during experimental brain tumour therapies with vectors that allow both targeting of the tumour, such as ganciclovir-induced activation of herpes simplex virus thymidine kinase (HSV-tk), and imaging using PET modalities[68]. Furthermore, in cell therapies following introduction of autologous genetically modified haematopoietic stem cells, the integration site of the genetic vector itself is a useful reporter to study the distribution and the persistence of grafted cells.

Traditional strategies for assessing graft function include electrophysiology, systemic measurements of hormone release and the use of *in vivo* microdialysis to determine neurotransmitter levels[69]. Recent tools of increasing importance include optogenetic reporters, which allow manipulation of neuronal activity in grafted cells through extrinsic light pulses. These reporters have been used to show connectivity of hPSC-derived neurons[70]. Similarly, the use of genetically encoded GCaMP3 calcium sensors was valuable in monitoring functional coupling of hPSC-derived cardiomyocytes in the host heart[4]. Genetic ablation studies are also important for determining the mechanisms that underlie graft function, such as by distinguishing short-term trophic effects from effects that are due to long-term functional engraftment. Suicide genes, such as those encoding HSV-tk and diphtheria toxin, have been successfully used to demonstrate a specific role for grafted neural stem cells in spinal cord injury models[71]. These genes have also been proposed as clinical fail-safe mechanism in case of over proliferation of grafted cells. More recent examples include the use of induced caspase-9, which is a trigger that is suitable for clinical use[72] and for eliminating both proliferating and postmitotic cells. Genetic engineering is likely to have important roles beyond safety switches to keep 'wayward cells' in check. Genetically modified cells can deliver therapeutically relevant proteins; for example, neural precursors that are genetically engineered to express glial cell line-derived neurotrophic factor (GDNF) are currently being developed as a therapeutic strategy for treating patients with amyotrophic lateral sclerosis[73]. Other important applications of genetically engineered stem cells include repair of a disease-causing gene, such as the replacement of β-globin in thalassemia[74], as well as the modulation of immunogenicity of the grafted cells or of the ability to express factors that could promote graft integration. A recent example for modulating graft integration and axonal outgrowth is the use of hESC-derived dopamine neurons that are modified to express PST (also known as SIA8D), which is the enzyme required to regulate polysialylated neuronal cell adhesion molecules at the cell surface[75].

Autologous cell sources

Patient-specific iPSCs are attractive as an essentially autologous source that should obviate the need for immunosuppression in the recipient, although this issue remains somewhat

controversial on the basis of studies in mouse iPSCs[76–78]. Full immunocompatibility is difficult to assess for human cells because of the lack of an experimental autologous grafting paradigm. A potential concern for using human iPSCs is the acquisition of genetic and epigenetic alterations during reprogramming, including mutations in protein-coding regions[79] and evidence of aberrant DNA methylation[80]. However, the presence of genetic and epigenetic aberrations is not limited to human iPSCs and to cellular reprogramming but is a concern for any cell-based therapeutic product.

Recent advances in reprogramming techniques obviate the need for integrating vectors[81–83], thereby eliminating the risk of reactivation of oncogenic reprogramming factors. Another concern that is specific to the use of iPSC-derived cells is whether reprogrammed cells retain an epigenetic 'memory'. There is clear evidence that both mouse and human iPSCs retain, at least transiently, an epigenetic marker profile that is partly related to the donor cell of origin and that can affect subsequent differentiation results[84–87]. Future studies will have to address whether the simple maintenance of iPSCs at the pluripotent stage can fully eliminate epigenetic memory, whether additional pharmacological or genetic interventions are required for full reset and whether any translational application of iPSC-derived lineages in cell therapy will require a careful analysis of the effect of donor cell origin on cell differentiation and function.

Some of the biggest practical hurdles for using patient-specific iPSCs are regulatory requirements, as well as the cost and timeframes that are associated with approving unique iPSC-based products for each individual. Given the current technologies and the existing regulatory framework, the costs that are associated with such a personalized treatment approach would be prohibitive, which makes it unlikely for such approach to enter routine, reimbursable medical practice. Both technological and regulatory changes will be required to overcome these important hurdles in the future. Nonetheless, researchers in Japan are currently seeking regulatory approval for the first of such study using patient-specific iPSC-derived RPE cells for the treatment of macular degeneration[88]. The recent breakthrough in establishing patient-matched hPSCs through nuclear transfer using a Dolly-like cloning approach[89] puts another potential cell source back into play for the development of patient-matched therapies through nuclear transfer of hESCs.

Directed conversion of somatic cells through the introduction of fate-specific transcription factors has been reported for several fates as reviewed recently[8,90]. However, the translational potential of this approach, including the ability for long-term functional engraftment, remains to be determined. The scalability and the establishment of a good manufacturing practice (GMP)-compliant cell therapy platform represent additional major hurdles towards clinical translation[91]. Another future research direction involves *in vivo* reprogramming with a goal of targeting the body's own cells. These efforts are currently limited to early preclinical studies, such as the conversion of different neuronal sub-types[92,93], the conversion of astrocytes into neurons[94] or neuroblasts[95], the *in vivo* conversion of exocrine to endocrine pancreatic cells[96] and the *in vivo* conversion of cardiac fibroblasts into cardiomyocytes[97]. All of these applications hold substantial promise for the future, although none of them seems to be close to clinical translation.

Next steps towards translation

In the past few years, there has been a strong drive towards translating hESC research into the clinic. The first of such attempt was the use of hESC-derived oligodendrocyte precursors for treating spinal cord injury with the goal of remyelinating denuded axons at the site of injury. The trial, which was sponsored by Geron, has pioneered the application of hESCs and illustrated the challenges for passing the regulatory hurdles in this new class of therapy. Although the spinal cord trial has been abandoned owing to financial and strategic considerations at Geron, the lack of major adverse effects in the four patients treated offers preliminary safety data for the approach[98]. The second application for a hESC-derived product was the use of RPE cells for treating eye disorders such as Stargardt disease — a juvenile form of macular degeneration — and age-related macular degeneration. These ongoing studies have reported survival of the cells in at least one of the patients who was initially grafted and associated improvement in visual function[47].

Beyond these few initial attempts, realizing the therapeutic potential of stem cells for clinical applications remains a central goal for the scientific community and the public. Two of the key elements for any successful translational application (FIG. 4) are the ability to produce hPSC derivatives in a scalable and GMP-compliant manner and, crucially, the selection of appropriate disease targets. Substantial progress has been made in the area of scalable production of hESC progeny using xenofree reagents, defined culture media and robust cryopreservation techniques, all of which were carried out under GMP-compliant conditions[99].

Diseases that are caused by the selective loss of specific cell populations have been at the forefront of clinical developments. However, precise patient selection often remains a major concern. In the case of Parkinson's disease[40], incomplete knowledge of the pathophysiology and of the potential clinical and molecular subsets of the disease is compounded by poorly representative animal models. Thus, improved phenotyping of patients using physiological, genetic and imaging approaches is crucial for selecting appropriate subjects for cell therapy. Furthermore, any cell-based approach has to be competitive with existing therapies, such as novel pharmacological agents, or with alternative surgical interventions such as deep brain stimulation[100].

Grafting techniques will benefit from remarkable technical innovations in the surgical field, such as the development of robotic platforms and minimally invasive delivery systems. For example, for intracranial injections, recent advances include the development of surgical technologies that provide real-time MRI guidance and visualization of the brain target[101]. The ability to monitor grafted cells could be coupled with non-invasive methods to control graft behaviour, such as the inducible safety switches discussed above. Finally, methods are under development to enhance *in vivo* cell migration and graft integration. Implementing hPSC-based approaches in regenerative medicine will require multidisciplinary teams of clinicians and scientists with expertise in directed differentiation, GMP production, large animal studies, tissue engineering and trial design, as well as ethicists and patient advocates. In addition, navigating the complex web of federal regulations requires specific expertise, especially in view of the pioneer status of these approaches. In fact, the Center for Biologics

Evaluation and Research of the US Food and Drug Administration (FDA) has recently published a guidance[102] entitled "Considerations for the design of early-phase clinical trials of cellular and gene therapy products", in which the FDA expressed concerns over the potential risks of extended biological activity, induction of immunogenicity and the involvement of relatively invasive procedures and devices for delivery.

Conclusion

Stem cell research has moved from an era of optimizing hPSC isolation to ongoing or imminent early-stage clinical trials. A major challenge that remains is to define a more comprehensive set of human disorders that may benefit from hPSC-based approaches. Furthermore, directed differentiation will have to include methods to control cell maturation, in addition to cell-fate specification, in order to access a full range of cell types and stages for applications in regenerative medicine. Finally, safety concerns remain an important issue despite the promise of preclinical studies. After 15 years of hESC research and six years since the isolation of human iPSCs, we are on the verge of defining the clinical use of hPSCs in human disease.

Acknowledgments

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Glossary

Directed differentiation	A method to control the differentiation of pluripotent stem cells into specific cell types, which is typically achieved by providing cells with extrinsic signals in a precise temporal sequence that mimicks development.

|	
Morphogen	A substance that is active in pattern formation and that varies in spatial concentration or activity; cells respond differently at different threshold concentrations of morphogens.
Embryoid body	A clump of cells that arises when embryonic cells are cultured and differentiated in suspension and that can give rise to cell types from all three germ layers (that is, the endoderm, mesoderm and ectoderm).
Stromal	Pertaining to connective tissue that is made up of both cells (such as fibroblasts) and matrix (such as collagen).
Dopamine neuron	A nerve cell that uses the neurotransmitter dopamine; those in the midbrain are affected in Parkinson's disease.
Dual SMAD inhibition	(dSMADi). The concomitant inhibition of bone morphogenetic protein and Nodal–activin–transforming growth factor-β signalling, which is used to obtain neural cells from human pluripotent stem cell sources.
Floor plate	A transient developmental structure along the midline of the embryo that is important for brain development.
Striatal neurons	Neurons that lie in the striatum, which is an area of the brain that is involved in fine movements, emotion and cognition.
Globus pallidus	A subcortical structure of the brain that is a major element of the basal ganglia system.
Oligodendrocytes	One of the three main cell types that make up the brain parenchyma, the other two being neurons and astrocytes. They produce myelin, which insulates axons to alter the conduction properties of neurons.
Self-organizing	Pertaining to an intrinsic programme in pluripotent stem cell-derived lineages that enables cells *in vitro* to assemble into tissue-like and organoid structures.
Transcription activator-like effector nucleases	(TALENs). Fusions of truncated TALEs to a nonspecific DNA-cleavage domain of the *Fok*I endonuclease. Each TALE contains an amino terminus, a custom-designed DNA-binding domain and a carboxyl terminus with the activation domain being removed.
.Position emission tomography	(PET). An imaging technique that detects the emission of positrons from the brain after a small amount of radioactive isotopes have been injected into the blood stream; it is used to quantitatively measure metabolic, biochemical and functional activity in living tissues.

Good manufacturing practice

(GMP). A set of standardized production and testing conditions that are required for developing a clinical-grade cell product and for obtaining regulatory approval for trials in human subjects.
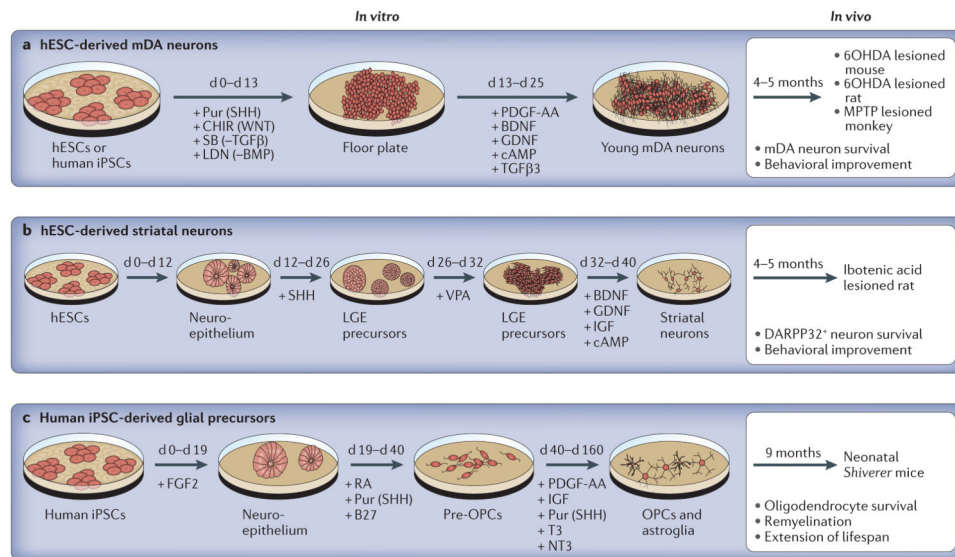
Figure 1. Generation of therapeutically relevant neural lineages from hPSCs

Schematic diagrams show published protocols for the generation of midbrain dopamine (mDA) neurons for the potential treatment of Parkinson's disease, striatal neurons for the treatment of Huntington's disease and glial precursors for the treatment of demyelinating disorders. Small molecules and growth factors that are used to direct cell fate are indicated below the arrows; the factors that are induced or inhibited are shown in parentheses. All studies showed robust long-term *in vivo* survival and functional improvement in at least one relevant animal model of disease. Although protocols for generating mDA neurons are relatively fast and efficient, protocols for generating oligodendrocyte precursor cells (OPCs) can take more than five months of *in vitro* differentiation. **a**. To differentiate human pluripotent stem cells (hPSCs) into mDA neurons through a floor plate intermediate[1], combined induction of neural floor plate using four small molecules from day 0 to day 13 (d 0–d 13) is followed by neuronal differentiation in the presence of a 'cocktail' of growth factors that promote mDA neuron fate. Cells are ready for transplantation into rodent or primate models of Parkinson's disease by day 25 of differentiation, which corresponds to the stage at which mDA neurons are born but still immature. **b.** To differentiate human embryonic stem cells (hESCs) into striatal neurons[2], neural induction into the neural rosette stage (that is, the neuroepithelium) is followed by exposure to a precise concentration of sonic hedgehog (SHH), which is required to specifically induce striatal precursors that emerge from the lateral ganglionic eminence (LGE) region during development. Floating cultures of LGE precursors are replated and further matured into striatal neurons. By day 40, these neurons are transplanted into a striatal lesion model of Huntington's disease. DARPP32 is a marker for spiny neurons. **c.** To differentiate human induced pluripotent stem cells (iPSCs) into glial precursors[3], neuroepithelial cells are isolated and treated with factors that promote ventral cell fates, followed by a protracted period of *in vitro* proliferation and maturation to obtain OPCs that are capable of efficient *in vivo* myelination. 6OHDA, 6-hydroxydopamine; BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; cAMP, cyclic AMP; CHIR, CHIR99021 (a small-molecule inhibitor); FGF2, fibroblast growth factor 2; GDNF, glial cell-derived neurotrophic factor; IGF, insulinlike

growth factor; LDN, LDN193189 (a small-molecule inhibitor); MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NT3, neurotrophin 3; PDGF-AA, platelet-derived growth factor AA; pur, puromycin; RA, retinoic acid; SB, SB431542 (a small-molecule inhibitor); T3, tri-iodothyronine; TGFβ, transforming growth factor-β; VPA, valproic acid.
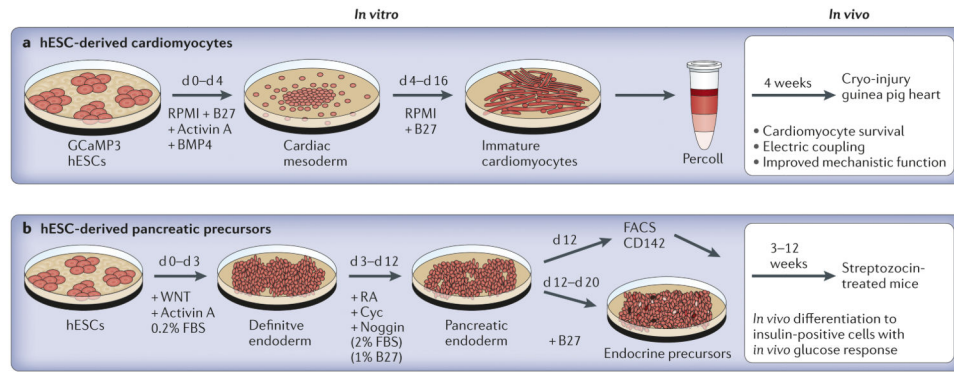
Figure 2. Generation of mesodermal and endodermal lineages from hESCs

Schematic diagrams show published protocols for generating cardiomyocytes as an example of a mesodermal lineage with therapeutic potential and for generating pancreatic precursors as a key endodermal derivative that is crucial for clinical translation. Small molecules and growth factors that are used to direct cell fate are indicated below the arrows. Protocols for both lineages are rather short and require an enrichment step before transplantation. **a.** In the generation of cardiomyocytes from human embryonic stem cells (hESCs)[4], a critical step is the induction of cardiac mesoderm in the presence of molecules that activate both bone morphogenetic protein (BMP) and Nodal– activin–transforming growth factor-β (TGFβ) signalling. Immature cardiomyocytes emerge by day 10 (d 10) of differentiation and are isolated by day 16. They are purified using Percoll, which is a physical separation technique. A critical step for the generation of cardiac cells is to show their ability to electrically couple with the host heart, as demonstrated using a genetic indicator of calcium signalling (GCaMP3). **b.** In the generation of hESC-derived pancreatic precursors[30,38], induction of the endoderm is achieved in the presence of molecules that trigger WNT and Nodal–activin–TGFβ signalling pathways. Pancreatic endoderm is obtained by day 12 of differentiation, the stage at which cells can be either further matured *in vitro* or directly transplanted *in vivo*. Although *in vivo* maturation of CD142+ cells results in a large number of insulin-positive cells with appropriate glucose response, current techniques for *in vitro* maturation do not yield cells with proper glucose responsiveness. Cyc, cyclopamine; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; RA, retinoic acid.
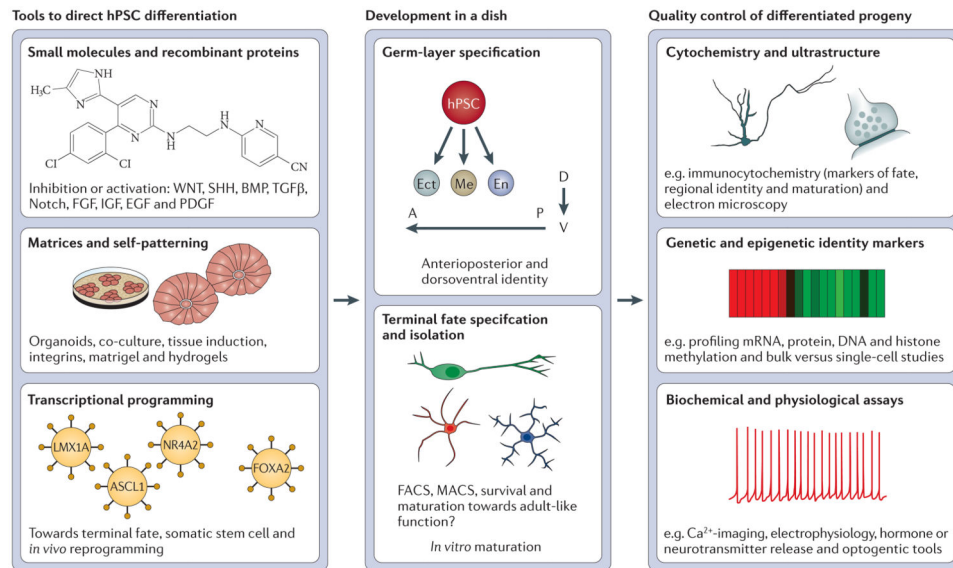
Figure 3. Directing fate and validating identity of hPSC-derived lineages

The key tools that are used to direct the differentiation of human pluripotent stem cells (hPSCs) to somatic cell types are shown. The use of small-molecule-based approaches has been particularly useful in generating cells with potential therapeutic relevance. Most strategies for directed cell differentiation are based on recreating aspects of normal development *in vitro*. One key developmental decision during the differentiation of hPSCs is the initial specification towards one of the three germ layer derivatives (that is, lineages of the ectoderm (ect), endoderm (en) or mesoderm (me)). A second crucial decision for hPSCs is to acquire specific anteriorposterior (A–P) and dorsoventral (D–V) patterning fates. By recreating those specific signalling conditions, it is possible to generate hPSC-derived lineages that correspond to the cells that originate in the different regions of the developing embryo. However, despite such progress, a limited understanding of human development and its protracted timeframes remain important 'roadblocks' in the field. Assessment of the authenticity of cell fate in the differentiated progeny is a crucial element of any directed differentiation strategy. In addition to traditional methods such as cytochemistry and gene expression studies, there is an increased need for robust *in vitro* functional assays. ASCL1, achaete-scute homologue 1; BMP, bone morphogenetic protein; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorting; FGF, fibroblast growth factor; FOXA2, forkhead box protein A2; IGF, insulin-like growth factor; LMX1A, LIM homeobox transcription factor 1-α; MACS, magnetic activated cell sorting; NR4A2, nuclear receptor subfamily 4 group A member 2; PDGF, platelet-derived growth factor; SHH, sonic hedgehog; TGFβ, transforming growth factor-β.
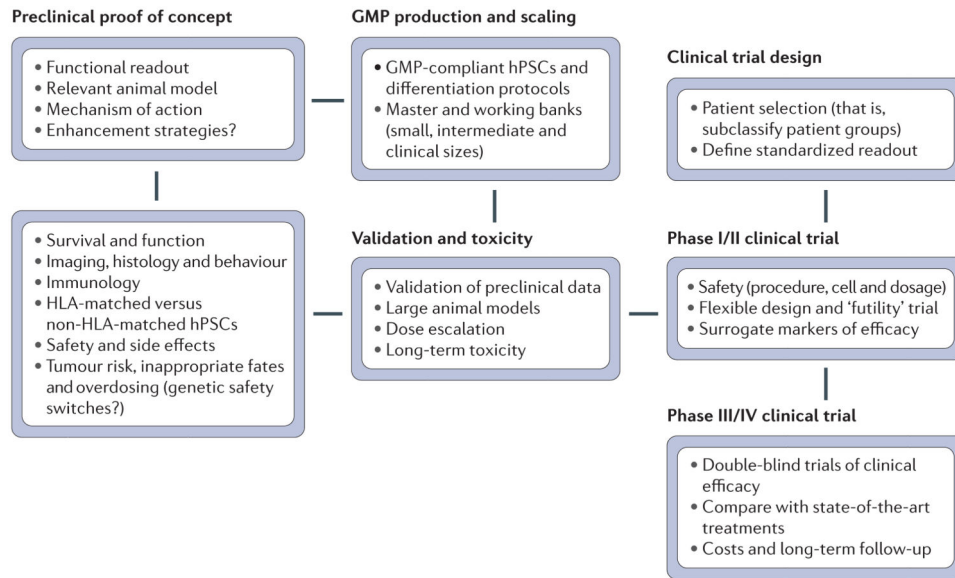
Figure 4. A roadmap towards clinical translation

To move human pluripotent stem cell (hPSC)-derived cells from the bench to clinical studies, some key steps and challenges need to be addressed. Following a preclinical proof of concept, it is important to validate robustness in relevant disease models and to develop a cell-manufacturing strategy that is suitable for clinical translation. Important steps include producing cells at a sufficient scale (that is, creating a cell bank) and using fully standardized protocols under current good manufacturing practice (GMP)-compliant conditions. The resulting bank of the candidate therapeutic cell product needs to be revalidated for safety and efficacy before it can be considered as a candidate clinical product for early safety studies, and eventually efficacy studies, in human patients. HLA, human leukocyte antigen.