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REVIEW

# Human pluripotent stem cells in regenerative medicine: where do we stand?

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## Abstract

**Human pluripotent stem cells have the capacity to self-renew indefinitely and the ability to differentiate into all cell types of a human body. These characteristics instill them with an enormous promise in regenerative medicine, where they could be used in cell, tissue and even organ-based replacement therapy. In this review, we discuss their potential clinical applications and the advantages and pitfalls for the different types of human pluripotent stem cells to transition from the bench to the bedside. We provide an overview of the current clinical trials, and the specific challenges we are still facing, including immune compatibility, suboptimal differentiation, risk of tumor formation and genome instability.**

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Human pluripotent stem cells (hPSC) have an unlimited capacity to self-renew and can differentiate into cells of all three embryonic germ layers, making them very appealing to the fields of regenerative medicine and *in vitro* disease modeling. There are two types of hPSC: human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC). The first are derived from early human embryos, while the second are obtained by reprogramming of somatic cells. By now, the derivation and culture of hPSC have found its way into many laboratories worldwide (Löser *et al.* 2010, Fraga *et al.* 2011), and these cells have become the basis of a very active research domain. The European Human Embryonic Stem Cell Registry contains 738 hESC and 1.822 hiPSC lines from 251 providers of 29 countries (http://www.hescreg.eu; Monthly Statistic Report To 01.08.2018), and, for instance, a PubMed search using the term 'human pluripotent stem cells' on 8/08/2018, yielded 27.179 results.

## The first steps of hPSC in regenerative medicine

The greatest therapeutic promise of hPSC is to cure individuals by replacement of somatic cells that were lost as a result of degenerative disorders or injury (Fig. 1). To date, hPSCs have been successfully differentiated into virtually all cell types, with the exception of functional gametes, and are being considered for the treatment of numerous conditions, such as neurodegenerative diseases, macular degeneration, cardiac failure and type I diabetes mellitus (Trounson & DeWitt 2016). Tissues and organs with a more complex organization are more difficult to obtain, although different groups have developed hPSC-derived organoid cultures that recapitulate the inner ear, retina, brain, lung, kidney, pituitary gland, liver, small intestine and stomach (reviewed in Kretzschmar & Clevers 2016). Of these, some of the most remarkable examples are the organoids with human brain structures (Lancaster *et al.* 2013, Renner *et al.* 2017), the renal tissues with a kidney-like organization (Takasato *et al.* 2014, Morizane *et al.* 2015) and the retinal organoids (DiStefano *et al.* 2018, Li *et al.* 2018, Mclelland *et al.* 2018). Nevertheless, although these developments are very exciting, the clinical translation of hPSC is just kicking off. Table 1 provides an overview of the ongoing and completed clinical trials using hPSC-derived cell types.

Neural cell types were among the first to be obtained through directed differentiation of hESC. The first clinical trial of hPSC was a safety trial using hESC-derived oligodendrocyte progenitor cells to treat spinal cord injury. It was initiated in October 2010 by the Geron Corporation and halted 1 year later due to a change in business strategy, after transplantation of five patients who were either paraplegic or quadriplegic. This clinical trial was later taken over by Asterias Biotherapeutics Inc. and completed. The therapy caused no adverse events, but also no motor or sensory neurological improvements were observed (Ilic *et al.* 2015). More recently, the company initiated a new phase 1/2a dose escalation study using the oligodendrocyte progenitor cells in patients with cervical spinal cord injury. Last


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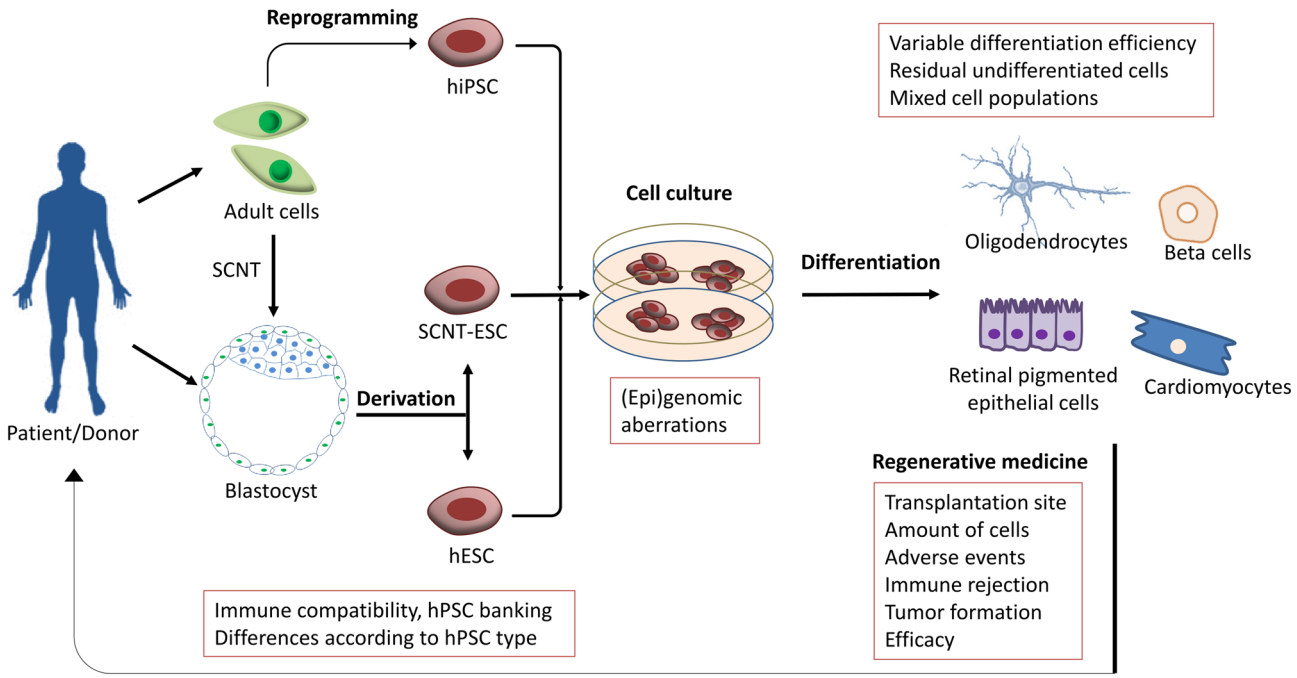


**Figure 1** The potential applications of human pluripotent stem cells and the challenges in bringing them from the bench to the bedside. The figure illustrates the different steps in the process, from the derivation of hPSC lines to the transplantation of their differentiated progeny. HPSC can be obtained from donated human blastocysts (human embryonic stem cells, hESC), from blastocysts obtained by somatic cell nuclear transfer (SCNT-ESC) and by somatic cell reprogramming (induced pluripotent stem cells, hiPSC). These last two types are by definition immune compatible with the donor of the somatic cells. The cells are then kept in culture to obtain sufficient cells to initiate differentiation. The terminally differentiated cells may be used in regenerative medicine. The main current challenges in this process are indicated in red boxes. These include the choice among the different hPSC and immune compatibility, the susceptibility of hPSC to genome instability during prolonged culture, suboptimal differentiation and the complications arising during regenerative medicine.

year, the company reported that 9 months after the treatment, three out of six of their patients showed motor level recovery of at least two levels. Patients without the treatment show comparable spontaneous recovery in 26% of cases (http://asteriasbiotherapeutics.com/).

The vast majority of the currently ongoing hPSC-based clinical trials involve the use of retinal pigmented epithelial (RPE) cells derived from hESC, hiPSC and somatic cell nuclear transfer (SCNT)-ESC to restore or improve vision in patients suffering from retinal degenerative diseases including acute age-related macular degeneration (AMD) and Stargardt's macular dystrophy (SMD) (Table 1; Schwartz *et al.* 2012, 2015, Diniz *et al.* 2013, Song *et al.* 2015, Mandai *et al.* 2017). These diseases are characterized by a loss of RPE cells, which leads to the death of the photoreceptors causing loss of vision. The human eye has become the favored testing ground for hPSC-based therapies for several reasons. Particularly, the eye is an immunoprivileged site, it is easy to monitor and the current RPE differentiation protocols are very robust and yield a pure cell population.

In 2013, the Riken Center for Developmental Biology initiated the first clinical research involving the use of hiPSC-derived cells in humans. A first patient suffering from AMD was transplanted with RPEs generated using hiPSC derived from her own skin cells. Unfortunately, the study was suspended in 2015 due to the identification of potentially harmful mutations in the second candidate's hiPSC and the RPE cells derived from them (Mandai *et al.* 2017). The first successfully completed phase I clinical trial with hESC-derived cells concluded on September 2014 in England with the transplantation of hESC-derived RPE sheets into 18 patients, nine affected with AMD and nine with SMD. The clinical trial did not only show the feasibility and safety of the procedure but also revealed an increase in the sub-retinal pigmentation in 13 out of 18 treated patients (Schwartz *et al.* 2015), and an improvement in visual acuity of at least 15 letters in eight of 18 patients during the first year after surgery, with no adverse effects seen in any of them (Schwartz *et al.* 2015).

A third ectodermal cell type that is making its way into the clinical trials are stem cell-derived dopaminergic neurons and neural precursor cells for the treatment of Parkinson's disease. Parkinson's disease is a neurodegenerative disorder caused by the death of ventral mesencephalic dopaminergic neurons. Transplantation of fetal dopaminergic neurons in Parkinson's disease patients, although yielding variable success, has demonstrated the feasibility of cell-based therapy in this disease. Over the years, several groups have proven that

hPSC are a potentially sound source of this specific cell type, and several pre-clinical studies have been carried out in rodent and primate models (reviewed in Lindvall 2016). Very recently, the Chinese Academy of Sciences, in collaboration with the first affiliated hospital of Zhengzhou university, have started a phase I/II, open-label study to assess the safety and efficacy of striatum transplantation of hESC-derived neural precursor cells in patients with Parkinson's disease. Specifically, the study will enroll 50 patients for cell injection, administering a single dose of neural precursor cells by stereotaxic intra-striatal injection (Wang *et al.* 2018).

Human ESC-derived cardiomyocytes have been successfully obtained either by directed differentiation (Laflamme *et al.* 2007) or via embryoid bodies formation (Caspi *et al.* 2007). They are also being investigated for the clinical application to support heart regeneration, and pre-clinical studies in small-animal models such as mice and rats have shown favorable results (Caspi *et al.* 2007, Shiba *et al.* 2012). Using a non-human primate model of myocardial ischemia, Chong *et al.* reported that these cells appear to fully integrate and regenerate infarcted hearts (Chong *et al.* 2014). Menasché and coworkers started a phase I clinical trial for transplantation of purified $CD15^+$ $Isl\text{-}1^+$ hESC-derived cardiac progenitors to six patients. A median dose of 8.2 million of highly purified hESC-derived cardiovascular progenitors were embedded in a biocompatible fibrin patch and transplanted into the infarcted area of the heart of patients with severely impaired cardiac function when the patients undergo scheduled coronary artery bypass surgery or mitral valve procedures (Menasché *et al.* 2014). This clinical trial was recently completed and demonstrated the technical feasibility of producing clinical-grade hESC-derived cardiovascular progenitors and supports their short- and medium-term safety, thereby setting the grounds for adequately powered efficacy studies (Menasché *et al.* 2018).

Another application that has reached the clinical trials is the use of hESC-derived pancreatic beta cells or pancreatic precursors to treat individuals with type 1 diabetes. Different research groups have obtained pancreatic endoderm that can efficiently generate glucose-responsive, insulin-secreting endocrine cells after transplantation (Kroon *et al.* 2008, Sui *et al.* 2013, Kirk *et al.* 2014, Pagliuca *et al.* 2014). ViaCyte Inc., a biotech company working on the development of a stem cell therapy for treatment of type 1 diabetes, optimized a protocol for the scalable production of functional pancreatic progenitors from hESC (Schulz *et al.* 2012). Their approach is based on delivering pancreatic precursor cells subcutaneously in a device with a selectively porous cell-impermeable membrane. These precursor cells are designed to further differentiate and mature *in vivo* after surgical implantation, not only to fully functioning insulin-producing beta cells but also to other endocrine cell types that make up the normal human pancreatic islet (Kirk *et al.* 2014). An alternative approach is the generation of islet-like organoids from hESC that are functionally capable of glucose-responsive insulin secretion as well as therapeutic effects (Kim *et al.* 2016). Recently, Ameri and coworkers have identified GP2 as a specific marker of human pancreatic endoderm cells and demonstrated that $GP2^+$ pancreatic endoderm cells efficiently differentiate into glucose-responsive insulin-producing cells. Isolation of the target cells using this cell surface marker has a further advantage that it can help to eliminate the residual undifferentiated hESC, thus offering a safer route toward the manufacture of endocrine cells for therapy (Ameri *et al.* 2017).

## The challenging side of hPSC

Despite the first successes in closing the gap between the bench and bedside, there is still a number of significant hurdles to overcome for hPSC to reach their full and safe clinical potential. Figure 1 illustrates each challenge at the different steps from hPSC derivation to their transplantation to the patient. In the next sections, we discuss the most significant issues in detail.

### *Ethical issues*

The main reservation that has been raised against the use of hESC is based on ethical grounds because the derivation of these cells requires the use of human embryos (Rosner *et al.* 2014). Conversely, it is possible to obtain hESC from single blastomeres of cleavage-stage embryos, this being a potential embryo-sparing approach (Klimanskaya *et al.* 2006, Geens *et al.* 2009). In this setting, one blastomere is biopsied from the embryo in the same manner as done for pre-implantation genetic diagnosis. While this blastomere could be then used to establish a hESC line, the rest of the embryo could further develop and be transferred to the patient. On the other hand, although technically possible, this approach is practically inviable, because it requires patients undergoing a fertility treatment to agree to a blastomere biopsy on their embryos, potentially compromising their chances of becoming pregnant.

### *Immune compatibility: hESC, SCNT-ESC and hiPSC*

A second issue raised on the use of hESC for transplantation was the risk of immune rejection since they are allogeneic to the patients (Zhan *et al.* 2004, Simonson *et al.* 2015). Currently, there are two options to solve this problem. The first appeared with the development of cell reprogramming (Takahashi & Yamanaka 2006, Takahashi *et al.* 2007). Human iPSC are reprogrammed directly from patients' somatic cells, and thus circumvent embryo destruction to establish hPSC lines and generate autologous hPSC. The second approach is the use of SCNT to generate patient-specific

**Table 1** On-going and completed clinical trials using hPSC-derived cells.

| Trial number | Start | Location | Status | Number of patients | Summary of the study |
|---|---|---|---|---|---|
| NCT01217008 | 2010 | Asterias Biotherapeutics, Inc., USA | Completed | 5 | Phase I, safety study of GRNOPC1 in patients with neurologically complete, sub-acute, spinal cord injury |
| NCT01469832 | 2011 | Astellas Pharma Inc, UK | Completed | 12 | Phase I/II, safety study of sub-retinal transplantation of hESC-RPE in SMD patients |
| NCT01344993 | 2011 | Astellas Pharma Inc, USA | Completed | 13 | Phase I/II, safety study of sub-retinal transplantation of hESC-RPE in patients with advanced Dry AMD |
| NCT01345006 | 2011 | Astellas Pharma Inc, USA | Completed | 13 | Phase I/II, safety study of sub-retinal transplantation of hESC-RPE in SMD patients |
| NCT01674829 | 2012 | CHABiotech CO., Ltd, Korea | Recruiting | Estimated enrolment: 12 | Phase I/IIa, safety study of sub-retinal transplantation of hESC-RPE in patients with advanced Dry AMD |
| NCT01625559 | 2012 | CHABiotech CO., Ltd, Korea | Unknown | Estimated enrolment: 3 | Phase I, safety study of sub-retinal transplantation of hESC-RPE in SMD patients |
| NCT01691261 | 2012 | Pfizer, UK | Suspended | Estimated enrolment: 10 | Phase I, safety and feasibility study of implantation of hESC-RPE in patients with acute wet AMD and recent rapid vision decline |
| NCT02463344 | 2012 | Astellas Institute for Regenerative Medicine, USA | Active, not recruiting | 11 | Long-term follow up to a phase I/II, safety study of sub-retinal transplantation of hESC-RPE in advanced dry AMD |
| NCT02057900 | 2013 | Assistance Publique – Hôpitaux de Paris, France | Completed | 10 | Feasibility and safety study of transplantation of hESC-derived CD15+ Isl-1+ progenitors in severe heart failure |
| NCT02941991 | 2013 | Astellas Institute for Regenerative Medicine, UK | Active, not recruiting | 11 | Follow-up to 5 years of a phase I/II, safety study of sub-retinal transplantation of hESC-RPE in SMD patients |
| NCT02122159 | 2014 | University of California, Los Angeles, USA | Withdrawn | N/A | Phase I/II, safety study of sub-retinal transplantation of hESC-RPE in patients with geographic atrophy secondary to MMD |
| NCT02239354 | 2014 | ViaCyte, USA | Active, not recruiting | Estimated enrolment: 65 | Phase I/II study with two cohorts to evaluate the safety, tolerability, and efficacy of various doses of VC-01 combination product in subjects with type 1 diabetes mellitus |
| UMIN000011929 | 2014 | Highway program for realization of regenerative medicine and others, Japan | On hold | 2 | Phase 1 Clinical Trial for the treatment of wet AMD Using an autologous hiPSC-derived RPE |
| NCT02286089 | 2015 | Cell Cure Neurosciences Ltd., USA and Israel | Recruiting | Estimated enrolment: 24 | Phase I/IIa dose escalation safety and efficacy study of hESC-RPE transplanted subretinally in patients with advanced dry AMD |
| NCT02302157 | 2015 | Asterias Biotherapeutics, Inc., USA | Active, not recruiting | Estimated enrolment: 35 | A Phase 1/2a dose escalation study of AST-OPC1 in subjects with cervical sensorimotor complete spinal cord injury |
| NCT02445612 | 2015 | Astellas Pharma Inc, USA | Active, not recruiting | 13 | Long-term follow up of sub-retinal transplantation of hESC-RPE in SMD patients |
| NCT02463344 | 2015 | Astellas Pharma Inc, USA | Active, not recruiting | 11 | Long-term follow up to a phase I/II, open-label, multi-center, prospective study to determine the safety and tolerability of sub-retinal transplantation of hESC-RPE in patients with advanced dry AMD |
| NCT02464956 | 2015 | Moorfields Eye Hospital NHS Foundation Trust, UK | Unknown | Estimated enrolment: 10 | Feasibility of production of iPSC-derived RPE cells fulfilling regulatory requirements for transplantation in dry AMD |
| NCT02749734 | 2015 | Southwest Hospital, China | Active, not recruiting | Estimated enrolment: 15 | Clinical study of sub-retinal transplantation of hESC-RPE in AMD and SMD |
| NCT02903576 | 2015 | Federal University of São Paulo, Brazil | Recruiting | Estimated enrolment: 18 | Phase I/II clinical trial using stem cell-derived RPE implantation in patients with AMD, SMD and exudative AMD |
| NCT01691261 | 2015 | Pfizer, University College London, UK | Active, not recruiting | 2 | Phase I, safety and feasibility study of implantation of hESC-RPE in subjects with acute wet AMD and recent rapid vision decline |
| NCT02590692 | 2015 | Regenerative Patch Technologies, LLC, USA | Active, not recruiting | 11 | Phase I/IIa safety study of sub-retinal implantation of hESC-RPE on a polymeric substrate in subjects with advanced, dry AMD |
| NCT03305029 | 2016 | CHA University, Korea | Enrolling by invitation | Estimated enrolment: 3 | Safety study of sub-retinal transplantation of SCNT-hES-RPE cells in patients with advanced dry AMD |
| NCT03102138 | 2016 | Pfizer, UK | Active, not recruiting | 2 | Long-term safety follow up study following transplantation of hESC-RPE in subjects with acute wet AMD and recent rapid vision decline |

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|---|---|---|---|---|---|
| NCT03046407 | 2017 | Chinese Academy of Sciences<br>The First Affiliated Hospital of Zhengzhou University, China | Recruiting | Estimated enrolment: 10 | A Phase I/II safety study in the treatment of Dry AMD with hESC-RPE |
| NCT03119636 | 2017 | Chinese Academy of Sciences<br>The First Affiliated Hospital of Zhengzhou University, China | Recruiting | Estimated enrolment: 50 | A Phase I/II, safety and efficacy study of striatum transplantation of hESC-derived neural precursor cells in patients with Parkinson's disease |
| NCT02755428 | 2018 | Chinese Academy of Sciences<br>Beijing Tongren Hospital, China | Recruiting | Estimated enrolment: 10 | Safety and Efficacy of Sub-retinal Transplantation of hESC-RPE in patients with dry AMD |

AMD, age-related macular degeneration; hESC-RPE, human embryonic stem cell derived retinal pigmented epithelial cells; MMD, myopic macular degeneration; SCNT-hES-RPE, somatic cell nuclear transfer human embryonic stem cell-derived retinal pigmented epithelial cells; SMD, Stargardt's macular dystrophy; UK, United Kingdom; USA, United States of America.

hESC lines (Tachibana *et al.* 2013, Chung *et al.* 2014). In this context, SCNT is performed by transferring the nucleus of a somatic cell to an enucleated human oocyte and the resulting embryos are then used for the derivation of ESC (SCNT-ESC) lines. It is important to keep in mind that SCNT is technically very challenging, and the derivation of ESC lines from human SCNT embryos has only recently been achieved (Tachibana *et al.* 2013, Chung *et al.* 2014).

Despite the strong similarities between hESC, hiPSC and SCNT-ESC, there is still debate on whether these cells are indeed equivalent both in biological terms and suitability for clinical applications (reviewed in Wolf *et al.* 2017). There are two main issues being discussed, namely (i) the differences in transcriptional and epigenetic landscape and (ii) the potential differences in mutational burden between these cells. For instance, Ma and coworkers compared genetically matched sets of hiPSC and SCNT-ESC to hESC and showed that the DNA methylation and transcriptional signatures of SCNT-ESC and hESC were remarkably similar and at the same time distinct to those of hiPSC, suggesting that SCNT-ESC undergoes more complete reprogramming than hiPSC (Ma *et al.* 2014). Using a similar approach, Johannesson *et al.* reported that gene expression and DNA methylation profiles of SCNT-ESC and hiPSC are similar (Johannesson *et al.* 2014). These contradictory results could be possibly partly explained by the use of different donor cell types, reprogramming methods and even time in culture, as the reprogramming process takes longer than first thought, and the cell lines settle in their pluripotent state with time (reviewed in Yoshihara *et al.* 2017*a*).

### *The cost of autologous hPSC*

Despite the initial appeal of generating autologous hPSC for regenerative medicine, it is a non-neglectable issue that the generation of patient-specific hiPSC or SCNT-ESC would create a very expensive and lengthy treatment. This has made the use of hPSC banks for allogeneic treatment more appealing and realistic. In this line, scientists from Kyoto University have been establishing an hiPSC bank containing multiple clinical-grade hiPSC lines from donors homozygous for three human leukocyte antigens (HLA) loci: HLA-A, -B and -DR. This allows optimized matching with patients, reducing the degree of immune rejection of the allogeneic transplants (Masuda *et al.* 2014, Azuma & Yamanaka 2016). Different studies estimate that to offer an HLA-matched transplant to 90% of the Japanese population, a cell bank size of 50 (Nakatsuji *et al.* 2008) up to 140 (Okita *et al.* 2011) homozygous hiPSC lines would suffice. For a hESC bank, on the other hand, lines derived from 170 randomly selected donated embryos would provide at least one hESC line with a single mismatch at one locus or better match for 80% of patients (Nakajima *et al.* 2007).

### *Suboptimal differentiation*

Another important issue is that the vast majority of differentiation protocols are not yet fully optimized. Most differentiation protocols result in a heterogeneous population of cells, frequently with only a small fraction of the desired cell type (D'Amour *et al.* 2006, Mfopou *et al.* 2010, Efthymiou *et al.* 2014). Often, these protocols try to mimic the microenvironment provided by the embryo. However, our understanding of these processes is still incomplete, and despite the use of matrices and specific growth factors to support the cells, the difference between the 3D environment of the embryo and the culture dish is still large. A strategy to partially counter this problem would be to derive and transplant progenitor cells rather than the fully differentiated cells (Kirk *et al.* 2014), assuming that the *in vivo* environment will assist the final differentiation steps. A further complication is added by the fact that many differentiation protocols show a wide variation in efficiency depending on which hPSC line is used as starting material. The leading hypothesis for these biases is genetic and epigenetic differences between the hPSC lines (extensively reviewed in Keller *et al.* 2018).

The epigenetic factor has received particular attention in hiPSC, with the idea of epigenetic memory. This concept stems from a number of works that investigated the differences in differentiation capacity of hiPSC and described them to epigenetic marks inherited from the cells of origin and that had not been correctly reset during the reprogramming process (Bar-Nur *et al.* 2011, Kim *et al.* 2011, Ohi *et al.* 2011). However, current knowledge suggests that the reprogramming process itself is prone to inducing epigenetic abnormalities (Tiemann *et al.* 2016) and that the main factor influencing the differentiation propensity is not an epigenetic memory as such, but the (epi)-genetic background of the donor of the source cells (reviewed in Keller *et al.* 2018).

The state of pluripotency of PSC lines is also emerging as an important inducer of differentiation bias. In mouse, ESC can exist in two states depending on the development stage of the embryos used for the derivation. Mouse naïve or ground state ESCs are derived from pre-implantation embryos, while mouse primed-state ESC are derived from post-implantation embryos. These two mouse ESC types have very different characteristics, including a lower differentiation bias in mouse-naïve ESC. In the human, hESC are derived only from pre-implantation embryos, but molecular analyses show that hESC are more similar to mouse primed-state ESC than to mouse-naïve ESC (Tesar *et al.* 2007, Nichols & Smith 2009, Hanna *et al.* 2010). Currently, the derivation of human-naïve ESC is possible through different methods, including the ectopic overexpression of the transcription factors and the use of tailored culture systems (reviewed in Weinberger *et al.* 2016). The most important advantage to human-naïve ESC is that they should display stable self-renewal capacity and survival, allowing efficient cell expansion and differentiation, and show less differentiation bias (Honda *et al.* 2013, Duggal *et al.* 2015). However, recent studies have confronted this notion by showing that naïve hPSC display a reduction in differentiation capacity and an increase in differentiation bias compared to the primed hPSC (Lee *et al.* 2017, Warrier *et al.* 2017). Furthermore, naïve hPSC would fail to generate mature cells, in contrast to their primed counterparts (Warrier *et al.* 2017).

An important problem caused by imperfect differentiation is the persistence of residual undifferentiated cells that may lead to tumor formation upon transplantation (Fujikawa *et al.* 2005, Blum & Benvenisty 2008, Sui *et al.* 2013). Even small numbers of remaining undifferentiated cells can lead to tumor formation (Lee *et al.* 2009) or the growth of immature tissues (Roy *et al.* 2006), making the achievement of a pure differentiated population of desired cell type prior to transplantation prerequisite. Several different attempts have been made to reduce the frequency of teratoma formation after transplantation, such as eliminating the residual undifferentiated cells by flow cytometry (Tang *et al.* 2011, Quintanilla *et al.* 2014), extending the *in vitro* differentiation culture period (Brederlau *et al.* 2006, Doi *et al.* 2012), reducing the number of transplanted cells (Lee *et al.* 2009) or treating the cell suspensions with agents that are specifically cytotoxic to undifferentiated cells prior to transplantation (Choo *et al.* 2008, Ben-David *et al.* 2013, Lee *et al.* 2013, Rosner *et al.* 2014, Mitsui *et al.* 2015). Nevertheless, and despite these efforts to eliminate these cells from the final differentiated product, the best solution would be to identify and tackle the causes of why these residual cells stay undifferentiated.

### *Genome instability*

There is significant concern about the genetic and epigenetic integrity of hPSC. The increasing body of knowledge on this topic has shown that, up to now, it appears that any aspect of the genome of hPSC that is investigated, reveals forms *de novo* mutagenesis and instability.

It is by now well established that hPSCs that acquire chromosomal abnormalities that offer them with a selective advantage will outgrow and eventually take over the culture (reviewed in Lund *et al.* 2012, Nguyen *et al.* 2013). For example, more than 20% of the hPSC lines worldwide show a gain of a small region of 20q11.21 (Amps *et al.* 2011), which leads to overexpression of *BCL2L1* (Bcl-xL), inhibiting the mitochondrial apoptosis pathway and conferring a strong selective advantage to the mutant cells (Avery *et al.* 2013, Nguyen *et al.* 2014). Remarkably, the 20q11.21 amplification is also common in cancer (Scotto *et al.* 2008, Beroukhim *et al.* 2010, Tabach *et al.* 2011), illustrating the link between culture

adaptation and malignancy. For the other common chromosomal abnormalities appearing in hPSC after prolonged culture (such as gains of chromosomes 1, 12 and 17), the driver genes have not yet been conclusively established. The most compelling evidence is for the gain of chromosome 12, where the smallest common region of gain comprises the pluripotency regulator *NANOG* (Draper *et al.* 2004, Mayshar *et al.* 2010, Ben-David *et al.* 2014). HPSCs carrying this gain show decreased differentiation capacity and their gene expression profiles are similar to that of germ cell tumors and cells transgenically overexpressing *NANOG* (Ben-David *et al.* 2014).

Although the subject of much research, what causes and modulates the spontaneous mutagenesis of hPSC in culture has not yet been fully understood. It is known that hPSC undergo significant DNA damage in culture, resulting in different chromosomal gains and losses in individual cells, leading to mosaic cell populations (Jacobs *et al.* 2014), and it has been repeatedly suggested that hPSC culture conditions may play a significant role in this process. The two most investigated factors have been the method of passaging and the oxygen tension in the incubators. A number of reports have shown that aneuploidy more commonly appears when using enzymatic or chemical passaging methods (Buzzard *et al.* 2004, Draper *et al.* 2004, Ravi *et al.* 2005, Imreh *et al.* 2006), and that room oxygen tension results in an increase in spontaneous chromosome breaks and gross structural rearrangements (Forsyth *et al.* 2006, Lim *et al.* 2011). Recently, culture medium has been identified as a major influence on DNA damage and genomic instability (Jacobs *et al.* 2016, Bangalore *et al.* 2017). High-density culture in KnockOut Serum Replacement (KnockOut SR) containing media and on mouse feeders results in medium acidification, leading to increased DNA damage and chromosome abnormalities (Jacobs *et al.* 2016). Conversely, cells grown in mTeSR and Essential 8 media show higher levels of genotoxic stress and DNA sequence changes than their counterparts grown in KnockOut SR medium (Bangalore *et al.* 2017). Additionally, aneuploidy as such appears to also make the cells more prone to further genomic instability, due to DNA replication stress, resulting in defective chromosome condensation and segregation (Lamm *et al.* 2016).

Next to acquired chromosomal changes, hiPSC have two additional sources of loss of genetic integrity: the reprogramming process and the genetic variation in the source cells, originating from somatic mutagenesis (reviewed in Yoshihara *et al.* 2017*a*). The first reports on the appearance of *de novo* CNVs, mostly deletions, in early passage hiPSC suggested that these mutations appeared during reprogramming process and that they progressively disappeared from the culture due to their deleterious effect on the cells (Hussein *et al.* 2011, Laurent *et al.* 2011). Later work showed, though, that at least half of these already existed as low-frequency variants in the source cells (Abyzov *et al.* 2012). Point mutations have also been repeatedly reported in hiPSC (Gore *et al.* 2011, Ji *et al.* 2012, Young *et al.* 2012, Sugiura *et al.* 2014, Araki *et al.* 2017, Yoshihara *et al.* 2017*b*), and there is evidence that their genome has significantly higher point mutation rates as compared to that of hESC (Sugiura *et al.* 2014). Here again, the point mutations are suggested to have either pre-existed in the parental somatic cells or to be reprogramming associated (Gore *et al.* 2011, Ji *et al.* 2012, Young *et al.* 2012) and particularly occurring during the initiation step of cell lineage conversion (Sugiura *et al.* 2014, Araki *et al.* 2017, Yoshihara *et al.* 2017*b*). By all accounts, this significant level of point mutations is concerning, particularly when bearing in mind that both hESC and hiPSC, in the undifferentiated and differentiated state, have been recurrently found to carry *TP53* missense mutations (Merkle *et al.* 2017). The consequences of this in the setting of regenerative medicine are potentially very damaging, as illustrated by the halted RIKEN clinical trial, upon identifying undisclosed genetic variants in their hPSC-derived cells (Garber 2015).

Finally, a last aspect that highlights how the somatic origin of hiPSC hampers their genetic integrity, are the findings regarding their mitochondrial genome. HESC show few variants in their mitochondrial DNA (mtDNA), at relatively low heteroplasmic loads, and are either inherited, or acquired during prolonged *in vitro* culture, but rarely are of high pathogenic potential (Maitra *et al.* 2005, Zambelli *et al.* 2018). In contrast, hiPSCs can display very high heteroplasmic loads of pathogenic mutations. By now, several groups have shown that these variants are found in the source cells (Mah *et al.* 2011, Kang *et al.* 2016, Perales-Clemente *et al.* 2016, Zambelli *et al.* 2018), that they originate from somatic mutagenesis, and correlate to the age of the cell donor (Kang *et al.* 2016). Although it is unclear what the exact impact of these mutations is on the functionality of the cells, studies from the field of disease modeling have provided evidence that they may induce defects in differentiation capacity and functionality of hPSC and hPSC-derived cells (Cherry *et al.* 2013, Folmes *et al.* 2013, Hatakeyama *et al.* 2015).

Taken together, there is substantial evidence indicating a susceptibility of hPSC to acquire mutations analogous to those found in cancers. Culture conditions during derivation, expansion and differentiation should be further optimized in such way that genetic instability is minimized, and hPSC and their derivatives should be subjected to genome-wide analyses, especially before clinical application, to avoid transplantation of what could be pre-cancerous cells. It is also clear that there is a lack of systematic studies addressing the risk of these mutations, and there are no guidelines to which clinicians and researchers can adhere (Andrews *et al.* 2017). At the moment, the COSMIC (Catalogue

of Somatic Mutations In Cancer) database and the Shibata list from the Pharmaceuticals and Medical Devices Agency in Japan (http://www.pmda.go.jp/files/000152599.pdf#page=8) are the two suggested resources to predict the functionality of these variants and assess their potential impact on the safety of these cells.

### *Conclusions*

Although holding the great potential in regenerative medicine, there is still a big gap to be filled for hPSC to reach their full potential. This review has provided an overview on the potential clinical applications, and the advantages and drawbacks of the different types of hPSC to transition from the bench to the bedside. In addition, we have also discussed the specific challenges that still need to be overcome, including immune compatibility, suboptimal differentiation, risk of tumor formation and genome instability in order to bring hPSC closer to the clinic.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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