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REVIEW

Ten years of iPSC: clinical potential and advances in vitro hematopoietic differentiation

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Abstract Ten years have passed since the first publication announcing the generation of induced pluripotent stem cells (iPSCs). Issues related to ethics, immune rejection, and cell availability seemed to be solved following this breakthrough. The development of iPSC technology allows advances in in vitro cell differentiation for cell therapy purpose and other clinical applications. This review provides a perspective on the iPSC potential for cell therapies, particularly for hematological applications. We discuss the advances in in vitro hematopoietic differentiation, the possibilities to employ iPSC in hematology studies, and their potential clinical application in hematologic diseases. The generation of red blood cells and functional T cells and the genome editing technology applied to mutation correction are also covered. We highlight some of the requirements and obstacles to be overcome before translating these cells from research to the clinic, for instance, iPSC variability, genotoxicity, the differentiation process, and engraftment. Also, we evaluate the patent landscape and compile the clinical trials in the field of pluripotent stem cells. Currently, we know much more about iPSC than in 2006, but there are still challenges that must be solved. A greater understanding of molecular mechanisms underlying the generation of hematopoietic stem cells is necessary to produce suitable and transplantable hematopoietic stem progenitor cells from iPSC.

Keywords Blood cells · Clinical trials · Hematopoietic differentiation · Induced pluripotent stem cells · Patent landscape

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Introduction

Ten years have passed since the first publication announcing the generation of induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). In 2006, scientists at the University of Kyoto reprogrammed mouse fibroblasts to generate induced pluripotent stem cells. One year later, two reports demonstrated the generation of human iPSC for the first time (Takahashi et al. 2007; Yu et al. 2007). iPSCs are artificial cells and, unlike embryonic stem cells (ESCs), are not derived from human embryos. Their somatic origin is a more suitable source for human pluripotent stem cells (PSCs). Consequently, they are considered a valuable tool for research and are potentially useful for therapeutic applications in regenerative medicine.

iPSC research is developing rapidly. It has remarkably grown from 2006 to 2014 but, after 2014, the number of publications was maintained. Our analysis shows a progressive increase, between 2007 and 2012, in the number of publications employing iPSC for hematopoietic differentiation (Fig. 1a–c illustrates the progress that has been made in the development of iPSC and the major works that have marked hematopoietic in vitro differentiation).

Many efforts are being made to standardize cell-based therapies while advances in basic research continue to provide a complete understanding of iPSC safety and effectiveness. However, cell therapy is just one of the applications for these cells; the first steps for the use of iPSC in drug screening and diseases modeling are being taken. In this review, we discuss the advances in in vitro hematopoietic differentiation of induced pluripotent stem cells and some potential clinical applications. We highlight the requirements and obstacles in generating suitable cells; as well, we evaluate the patent landscape and compile the clinical trials in this field.

Advances in in vitro hematopoietic differentiation using iPSC as cell source

Fifteen years ago, scientists firstly reported the derivation of hematopoietic cells from human embryonic stem cell grown over a monolayer of S17 (murine stromal cell line) or C166 (yolk sac endothelial cell line) (Kaufman et al. 2001) (Fig. 1c). Years later, the in vitro production of hematopoietic progenitor cells (CD34+) was improved by co-culturing hESC with OP9 cells (a bone marrow stromal cell line derived from mice deficient in macrophage colony stimulating factor (M-CSF)) (Vodyanik 2005). Co-culture with OP9 cells does not require exogenous growth factors or complex embryoid structures. This system facilitates the study of molecular mechanisms involved in development and differentiation of hematopoietic cells. However, the co-culture system is inefficient to generate cells expressing CD45 (pan-leukocyte marker). Currently, many protocols for hematopoietic differentiation are based on the formation of embryoid bodies (EBs) with cytokine supplementation. EB recapitulates many aspects of embryogenesis. Probably, EB undergoes the transient in vitro gastrulation stage that precedes the expression of mesodermal

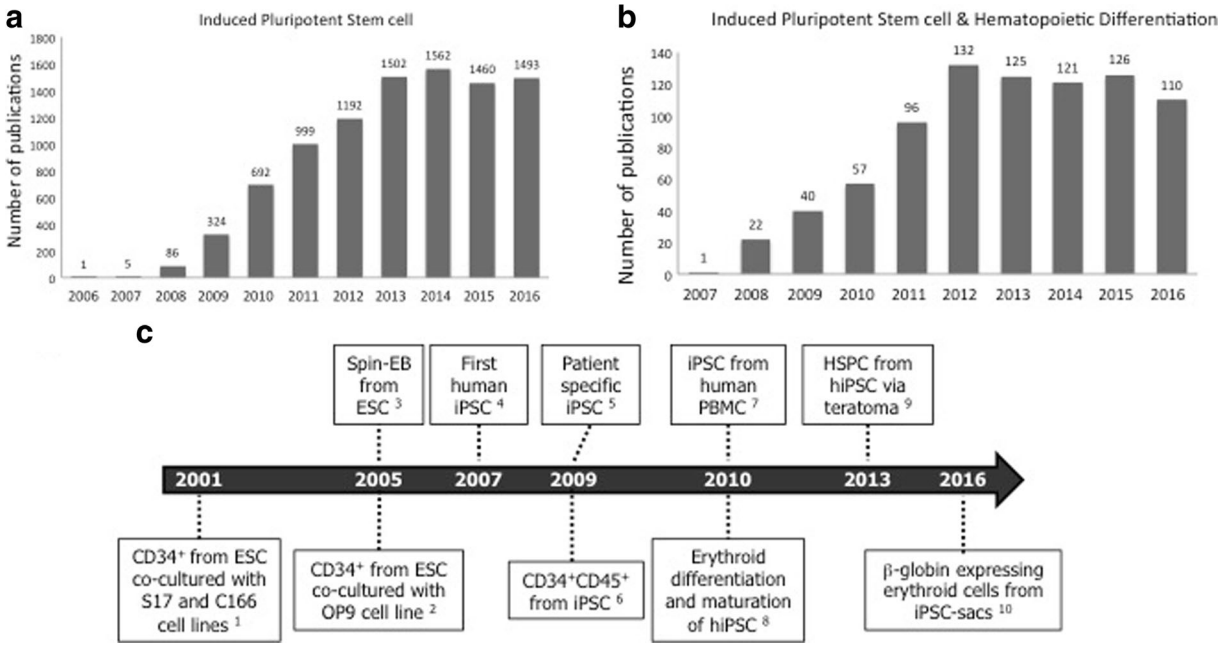

Fig. 1 **a** Global publication count (2006–2016*) for iPSC and **b** number of publications regarding iPSC and hematopoietic differentiation. Source: Scopus.*Analysis performed until December 8th, 2016. **c** Timeline comprising the major advances in iPSC and hematopoietic in vitro differentiation. *1*, Kaufman et al. (2001); *2*, Vodyanik et al. (2005); *3*, Ng et al. (2005); *4*, Takahashi et al. (2007); *5*, Loh et al. (2009); *6*, Lengerke et al. (2009); *7*, Loh et al. (2010), Staerk et al. (2010); *8*, Lapillonne et al. (2010); *9*, Amabile et al. (2013), Suzuki et al. (2013); *10*, Fujita et al. (2016)

genes, secreting hematopoietic cells (CD34+) (Keller et al. 1993; Lacaud 2002; Ng et al. 2008; Ng et al. 2005). Since its inception, iPSC have become an alternative source of pluripotent cells and many advances in vitro hematopoietic differentiation have been made. Lengerke et al. (2009) showed the robust generation of CD34+CD45+ cells from iPSC by in vitro differentiation, recapitulating aspects of early embryonic development. However, to date, it has not been shown that hematopoietic stem cells derived in vitro from iPSC are able of long-term engraftment in nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice. Studies pointed that HOX gene cluster plays a key role in the generation of long-term repopulating hematopoietic stem cells. In a hematopoietic differentiation model of murine iPSCs, it was demonstrated that long-term repopulating hematopoietic stem cells can be sustained in vitro by ectopic expression of HoxB4 (Izawa et al. 2014). The niche seems to be crucial for hematopoietic development. Two studies have shown the derivation of hematopoietic stem/progenitor cells (HSPC) within teratomas from human iPSC. The derived HSPC were able to reconstitute the entire hematopoietic system in NOD-SCID mice, also showing secondary repopulation capacity (Amabile et al. 2013; Suzuki et al. 2013).

The induction of a specific differentiation pathway by the addition of cytokines and/or co-culture of feeder cells is essential for generating mature cells, such as erythrocytes, monocytes, lymphocytes, and macrophages. For induction of the first stage of hematopoietic differentiation, most protocols add stem cell factor (SCF), bone morphogenetic protein 4 (BMP4), and vascular endothelial growth factor (VEGF) for mesodermal differentiation, while some protocols additionally use activin A to promote the early specification of hematopoietic fated mesoderm (Cerdan et al. 2012). In addition, special culture media have been developed, such as StemSpan, BPEL, and APEL (Ng et al. 2005; Olivier et al. 2006; Pick et al. 2013; Vanhee et al. 2015). The second stage of differentiation can be called maturation. For the production of erythrocytes from iPSC or ESC many studies, in addition to SCF, BMP4 and VEGF use Flt3, TPO, IL6, IL3, G-SCF, and EPO to promote hematopoietic differentiation inside embryoid bodies, resulting in the appearance of tissue-like structures such as blood islands and early blood vessels (Ye et al. 2009b).

Systems of in vitro differentiation try to mimic what occurs in vivo in a very simplified form. When erythroid cells are derived from ESC/iPSC by traditional in vitro differentiation protocols (EB formation and co-culture system), they mainly express embryonic type ε-globin, some fetal type γ-globin, and very little adult type β-globin. This indicates that in vitro differentiation does not correctly mimic what occurs in vivo. Perhaps, for the production of hematopoietic cells in vitro, the generation of hemangioblast during the differentiation process is indispensable. This hypothesis has been tested by Takayama et al. (2008), who showed that culture of hESC on stromal cells (C3H10T1/2 or OP9 cells) with vascular endothelial growth factor promoted the emergence of sac-like structures, named embryonic stem cell-derived sacs (ES-sacs), and improved the hematopoiesis. These ES-sacs consisted of multiple cysts demarcated by cellular monolayers that retained some of the properties of endothelial cells. Recently, Fujita et al. (2016) showed that iPSC sacs were able to derive erythroid cells with β-globin expression.

The difficulty in establishing in vitro models for differentiation and culture of hematopoietic progenitor cells (HPCs) is mainly due to the lack of full understanding of the complex process of hematopoietic differentiation that occurs in bone marrow microenvironment. The inability of these protocols to generate functional HPC with long-term engraftment potential may be related to the characteristics of the artificial microenvironment produced in culture systems not yet having all the necessary requirements to generate bona fide hematopoietic cells.

Potential applications of iPSC and derived blood cells

Induced pluripotent stem cells have the potential to generate many cell types that may have clinical applications. Here, we point out some of their main uses, especially the use of iPSC in disease modeling and drug screening; production of cells for cell therapy purpose, such as red blood cells for transfusion; and generation of modified cells such as CAR-T cells and mutation-corrected cells in hematological diseases (Fig. 2).

Disease modeling and platform for drug screening

Scientists often use disease modeling to investigate how a disease works at the molecular and cellular levels. It usually comprises the culturing of patient-derived cells,

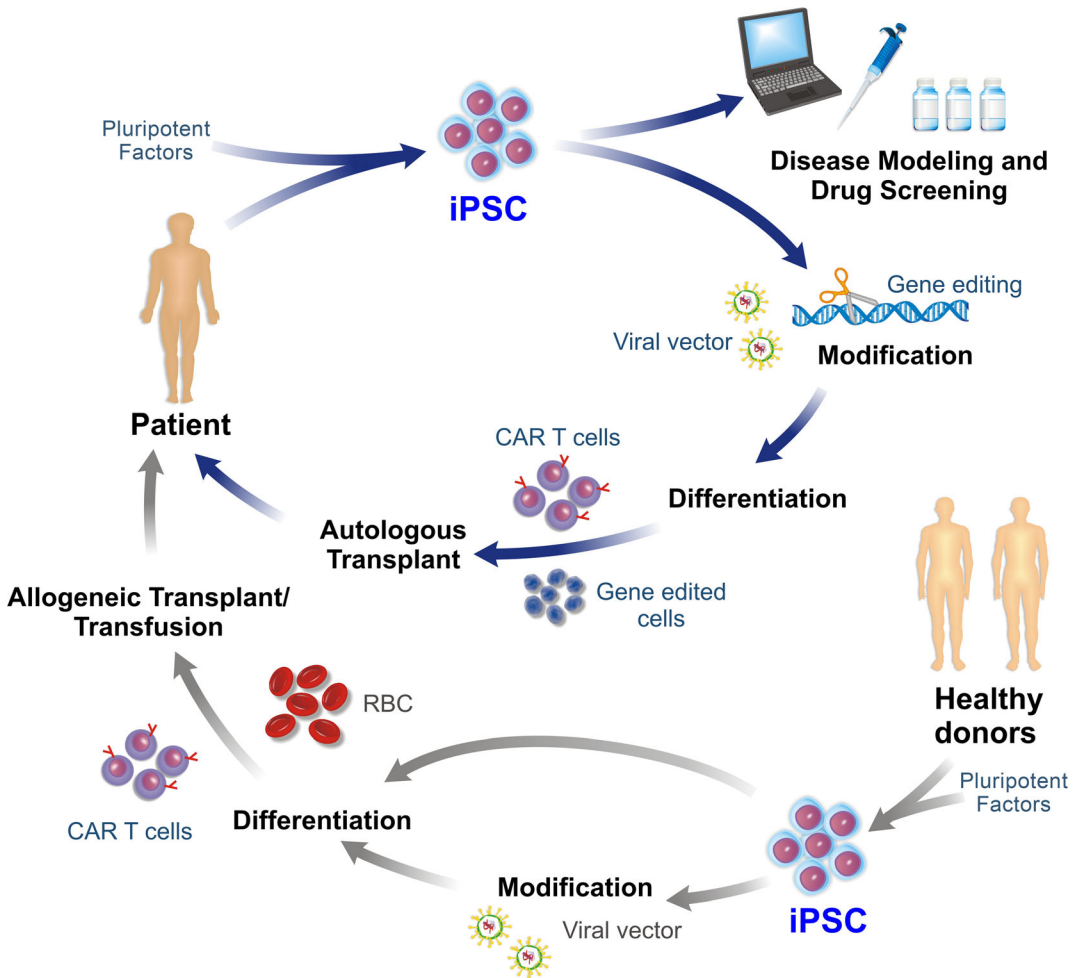

Fig. 2 Applications of iPSC in the fields of drug screening, disease modeling, and cell therapy. iPSC derived from patients' somatic cells (e.g., fibroblasts and PBMC) enable the generation of a large number of disease-affected cells for drug screening and disease modeling. iPSC can be an alternative source of cells for cell therapy. They can be cultured and differentiated into the desired cell type to be transplanted back into the patient. These cells can be modified, for example, to produce CAR T cells or have their genomes edited for correction of mutations. iPSC derived from healthy donors' somatic cells can be differentiated and modified to be used in patients, for instance, for the generation of universal RBC for transfusions and the production of CAR T cells

which can be immortalized or tumor cells. However, availability of diseased tissue limits this type of research. Another restriction is the fact that frequently only specific cell types express the disease phenotype, even the disease having a genetic background. The use of iPSC is an approach that can overcome such limitations. Human iPSCs are able to self-renew indefinitely and can be differentiated in vitro into any cell type. This way, researchers have in hands a powerful tool to generate a large number of disease-affected cells for either disease modeling or drug screening (Avior et al. 2016). If derived from patients' somatic cells, iPSC (Loh et al. 2009) will carry the molecular defect of interest and can be readily used for disease modeling of a variety of hematologic disorders (Table 1).

Besides disease modeling, the culture of disease-affected cells derived from iPSC can be used for drug screening. To identify novel therapeutic molecules in vitro, a high-throughput screening (HTS) is performed, in which cells are tested against a large number of substances. On the other hand, when potential drugs are available, two other strategies can be employed: candidate drug approach and patient-specific therapy. Contrary to HTS, these approaches use a small, refined library of potential therapeutic compounds (Avior et al. 2016). Compared to conventional drug discovery,

Table 1 iPSC for modeling of hematological diseases

Disease	Somatic cell for reprogramming	Reprogramming method	Known mutation(s)	Differentiated cell	Reference
β-Thalassemia	Skin fibroblasts, amniotic fluid cells, and chorionic villi	Retroviral vectors (Oct4, Sox2, Klf4, and cMyc)	4-bp deletion frameshift (−CTTT) in *HBB*	Fetal hemoglobin producing hematopoietic cells	Ye et al. (2009a)
Diamond-Blackfan Anemia	Skin fibroblasts and mononuclear cells	Lentiviral vectors or Sendai virus (Oct4, Sox2, Klf4, and cMyc)	Mutations in *RPS19* and *RPL5*	CD41+CD235+ primitive multilineage progenitor cells	Ge et al. (2015)
Chronic Myeloid Leukemia	CD34+ bone marrow cells	Retroviral vectors (Oct4, Sox2, Klf4, and cMyc)	*BCR-ABL* fusion	BCR-ABL expressing CD34+ hematopoietic progenitors	Kumano et al. (2012)
	CD34+ peripheral blood cells	Lentivectors (OSK1 and Mshp53)	*BCR-ABL* fusion	CD34+CD45+ hematopoietic cells	Bedel et al. (2013)
	Bone marrow mononuclear cells	Episomal vectors (Oct4, Sox2, Nanog, Lin28, cMyc, Klf4, and SV40LT)	*BCR-ABL* fusion	CD34+ leukemia stem cell-like cells	Suknuntha et al. (2015)
CAMT	Fibroblasts	Retroviral vectors (Oct4, Sox2, Klf4, and cMyc)	Loss of function or deletion of *MPL*	CD34+ hematopoietic cells	Hirata et al. (2013)
Dyskeratosis Congenita	Fibroblasts	Retroviral vectors (Oct4, Sox2, Klf4, and cMyc)	Deletions in *TERC*	*Not differentiated*	Agarwal et al. (2010)
FPD/AML	Peripheral T cells	Sendai virus (Oct3/4, Sox2, Klf4, and cMyc)	Mutations in *RUNX1*	CD34+CD45+ hematopoietic cells	Sakurai et al. (2014)
Fanconi Anemia	Skin fibroblasts and keratinocytes	Retroviral vectors (Oct4, Sox2, Klf4, and cMyc)	Mutations in *FANCA* and *FANCD2*	CD34+ cells	Raya et al. (2009)
Hemophilia A	Urinary cells	Episomal vectors (Oct4, Sox2, SV40LT, Klf4, and miRNA302–367)	Inversion of *F8* intron 22	FVIII deficient hepatocyte-like cells	Jia et al. (2014)
JMML	Mononuclear cells from bone marrow or peripheral blood	Lentiviral vectors (Oct4, Sox2, Klf4, and cMyc)	Missense mutations in *PTPN11*	Erythroid, myeloid, and megakaryocytic cells	Gandre-Babbe et al. (2013)
	Skin fibroblasts	Retroviral vectors (Oct3/4, Sox2, Klf4, and cMyc)	Missense mutations in *PTPN11*	CD33+ myeloid cells	Mulero-Navarro et al. (2015)
Myelofibrosis	CD34+ cells from bone marrow or peripheral blood	Retroviral vectors (Oct3/4, Sox2, Klf4, and cMyc)	Chromosome 13q deletions and JAK2V617F mutations	Megakaryocytes overexpressing interleukin-8	Hosoi et al. (2014)
Pearson Syndrome	Bone marrow-derived fibroblasts	Retroviral vectors (Oct3/4, Sox2, Klf4, and cMyc)	Deletions in mtDNA	Erythroid precursors with pathologic iron granule deposition	Cherry et al. (2013)
Severe Congenital Neutropenia	Skin fibroblasts	Retroviral vectors (Oct3/4, Sox2, Klf4, and cMyc)	Mutations in *HAX1*	Neutrophils	Morishima et al. (2014)
Shwachman-Diamond Syndrome	Bone marrow mesenchymal cells	Retroviral vectors (Oct4, Sox2, Klf4, and cMyc)	Mutations in *SBDS*	Pancreatic and hematopoietic cells	Tulpule et al. (2013)

CAMT congenital amegakaryocytic thrombocytopenia, *FPD/AML* familial platelet disorder/acute myeloid leukemia, *JMML* juvenile myelomonocytic leukemia

pluripotent cell-based drug screening offers a personalized therapy, reduced cost, and toxicity prediction, for example, in cardiac safety pharmacology assays (Braam et al. 2010; Sayed et al. 2016).

Cell therapy

Every year, the demand for blood products for patients suffering from a variety of hematological diseases and cancers constantly increases. However, patients who are chronically transfused can suffer from erythrocyte alloimmunization, infections, and iron overload (Ugwu et al. 2015; Yazdanbakhsh et al. 2012). Hence, RBCs derived from iPSC can represent an alternative source of cells for transfusions. The development of iPSC capable of producing universal erythrocytes is desired. The universal erythrocyte can be defined as an erythrocyte, which does not have any antigen on its surface. Taking into account the physiology of red blood cells, it is not possible to predict the existence or production of this universal erythrocyte. These blood antigens have fundamental functions, such as cell structure and are receptors of various substances. Even though the generation of a universal erythrocyte is unlikely at this time, it would be theoretically possible to generate several erythrocytes from different clones of iPSC, which are complementary regarding blood antigens and blood transfusion systems. Peyrard et al. (2011) showed that only 15 iPSC clones would be enough to represent the most frequently RBC phenotypes and it could treat 100% of alloimmunized patients (Kappler-Gratias et al. 2011). Certainly, the number of iPSC clones should vary with the level of miscegenation of each country.

The immunotherapy with T cells is a potentially effective strategy for the treatment of cancer and viral infections. However, the in vitro expansion of T cells has proved limited and often does not reach the amount necessary for clinical cell application. One way of overcoming this obstacle is using iPSC to produce effector T cells. However, its therapeutic potential needs to be proven (Kennedy et al. 2012; Timmermans et al. 2009; Vizcardo et al. 2013). The functional characterization of T cells derived from pluripotent cells is complicated. Rearrangement of T cell receptor (TCR) genes occurs randomly, so the cells generated in vitro have an unpredictable T cell receptor repertoire (Timmermans et al. 2009). An alternative would be to establish iPSC from mature cytotoxic T cells with TCRs already rearranged to a specific epitope (Vizcardo et al. 2013). Furthermore, as TCRs recognize antigens presented by specific HLA molecules, T cells from the iPSC need to match the HLA of the recipient patient for a successful clinical application. This problem can be solved by engineering the iPSC-derived T cells with an artificial antigen receptor, also known as chimeric antigen receptor (CAR) that acts independently of receptor HLA. Themeli et al. (2013) modified an iPSC line with CAR targeting the B cell antigen CD19. They demonstrated that these CAR-modified T cells exhibited in vitro and in vivo cytotoxic activity and specifically targeted CD19-expressing lymphoma cells. These results show that iPSC can give rise to functional T cells and may be used in cancer therapy in the future.

The genome editing technology allows the modification of specific genome locations (Fig. 3). The editing process is performed by special nucleases, such as zinc finger nucleases (ZFNs) (Scott 2005), transcription activator-like effector nucleases (TALEN), meganucleases (Hafez et al. 2012), and CRISPR/Cas systems (Gaj et al. 2013). These nucleases can recognize a specific sequence of the genome and create double strand breaks (DSBs). The targeting of DNA by ZFN and TALEN is done by DNA binding domains. Each ZFN domain recognizes a DNA triplet, while each TALEN domain recognizes a single nucleotide. CRISPR/Cas systems use a 20-nucleotide single guide RNA (sgRNA) strand for targeting a specific sequence of DNA (Gaj et al. 2013). Different from ZFN and TALEN, which are active as dimers having two *Fok*I endonuclease domains interacting to form a functional domain (Boch et al. 2009), the Cas9 endonuclease has a bi-lobed architecture harboring two nuclease domains, RuvC and HNH. RuvC cleaves the non-target DNA strand, while HNH cleaves the DNA targeted by the guide RNA (Sternberg et al. 2015). These breaks in DNA strands can be repaired (Fig. 3b) by the error-prone non-homologous end joining (NHEJ) in the absence of a repair template, resulting in deletions/insertions. When a repair template, which can be a double-stranded DNA or a single-stranded DNA oligonucleotide (ssODN), is available, the DSB is repaired by the high-fidelity homologous recombination (HR), resulting in the substitution of specific nucleotides (Ran et al. 2013).

Patients with a variety of hematologic conditions might benefit from these gene editing technologies, as their iPSC can be corrected and differentiated into the

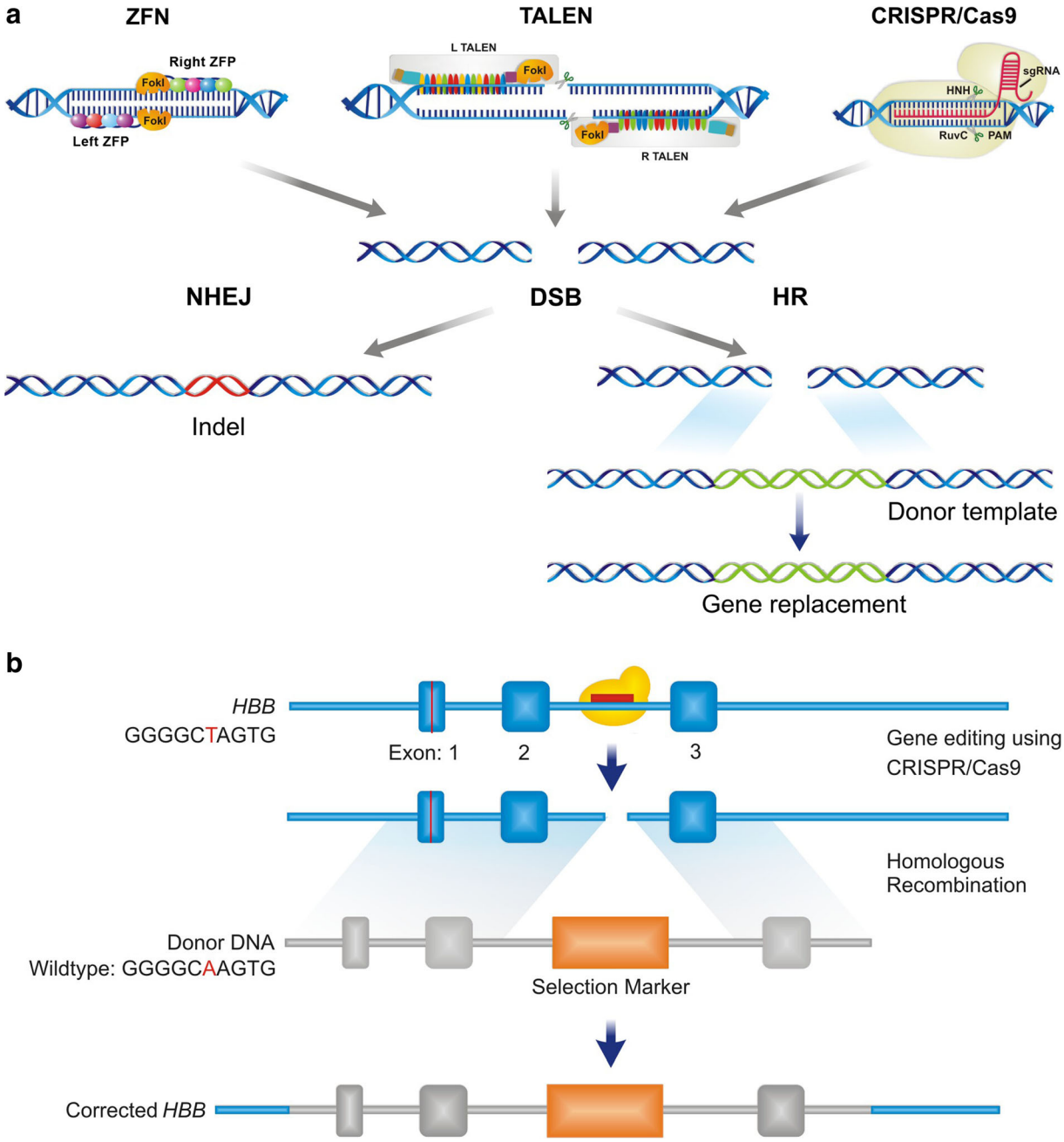

Fig. 3 Genome editing technologies. The genome editing technologies use nucleases that recognize and create double strand breaks (*DSB*s) to modify specific genome locations. **a** Zinc finger nucleases (*ZFN*s) are synthetic proteins consisting of an engineered zinc finger DNA-binding domain (zinc finger protein, *ZFP*) fused to the cleavage domain of the *Fok*I restriction endonuclease. ZFNs recognize target DNA sequences by virtue of three custom-designed zinc finger proteins each recognizing three nucleotides. Transcription activator-like effector nucleases (*TALEN*) are dimeric transcription factors/nucleases built from a set of 33 to 35 amino acid modules, each of which targets a single nucleotide. Hypervariable amino acids in the 12th and 13th positions (repeat-variable diresidue or RVD) are responsible for specific nucleotide targeting. In the CRISPR/Cas system, the Cas9 holoenzyme is directed to the target site by the guide RNA to cleave the DNA at a position close to the PAM motif. **b** CRISPR/Cas9-mediated gene correction of codon 17, A-T mutation β-globin gene (*HBB*) in β-thalassemia

desired cell type. Park et al. (2015) showed that it is possible to correct iPSC from hemophilic A patients containing large chromosomal rearrangements. The frequent inversions found in several hemophilic patients that involve introns 1 and 22 of the F8 gene were corrected using CRISPR/Cas9 nuclease without detectable off-target mutations in other genome locations. These cells were differentiated into endothelial cells and were able to correct hemophilia A in mice. iPSCs from β-thalassemia patients with mutations in the β-globin gene (*HBB*) were also efficiently corrected using CRISPR/Cas9 system (Huang et al. 2015; Niu et al. 2016; Song et al. 2015). Approaches to correct genetic defects in a wide range of hematologic disorders are shown in Table 2.

Requirements for generation of clinical grade cells

CGMP refers to the Current Good Manufacturing Practice regulations enforced by the US Food and Drug Administration (FDA), and the goal is to prevent instances of contamination, mix-ups, deviations, failures, and errors at a pharmaceutical company, assuring that the products meet quality standards (U.S. Food and Drug Administration 2015). Because stem cells and stem cells products have the potential for clinical application, their manufacturing process also need to follow the CGMP requirements. Generation of CGMP compliant cells from pluripotent stem cells has been reported. However, most of the cells were not fully derived under CGMP conditions (Baghbaderani et al. 2015). The FDA, to this date, has approved only one stem cell-based product, which is the use of cord blood-derived hematopoietic progenitor cells (U.S. Food and Drug Administration 2012). Many ethical, technological, and regulatory obstacles must be overcome before the implementation of iPSC-based clinical treatments (Simonson et al. 2015). Furthermore, to move from the research into the clinical application, stem cell and stem cell-based products will need to meet some requirements.

The generation of iPSC helped researchers to overcome the ethical concerns in regard to the source of pluripotent stem cells. Patient's somatic cells can be collected and reprogrammed into the pluripotent stage, generating iPSC, to be further differentiated and used by the same patient. This strategy also minimizes the immune response that exists when transplanting pluripotent stem cell (Minami and Murry 2007). Although, some researchers reported that iPSC are capable of inciting immune response in some cases (Araki et al. 2013; Todorova et al. 2016). To obtain somatic cells from patients, it is preferable a minimally invasive strategy. Many research groups have reported successful generation of iPSC from peripheral blood cells (Dowey et al. 2012; Loh et al. 2010; Ohmine et al. 2011; Staerk et al. 2010). To be suitable for research and clinical purposes, cell samples must be viable and free of contaminants, mycoplasma, and pathological microorganisms, besides being a homogenous population of desired cells. The source of the cells, the purification method, and the differentiation process might affect the culture, resulting in a multitude of cell types. Controlling the variability and maintaining only desired cells in culture is important to avoid residual cell types. When there is only the need for differentiated cells in the culture, terminal differentiation and elimination of residual pluripotent stem cells can be used. Another strategy is to enrich the culture for cells of interest through techniques of purification by either depleting pluripotent stem cells or by positively selecting only differentiated cells (Rodrigues et al. 2015). After enriching the cell population, it is necessary to determine the purity of the culture. A variety of methods, including quantitative polymerase chain reaction assay (qPCR), flow cytometry, and immunohistochemistry can be performed to evaluate the presence of markers of interest and the absence of those specific markers for undesired cells (Goldring et al. 2011).

To efficiently obtain iPSC, it is important to establish a robust, reproducible, and cGMP-compliant manufacturing process, which will allow cells to proliferate extensively, generating sufficient quantities for any possible clinical application (Baghbaderani et al. 2015). The original protocol for iPSC generation utilizes retroviral transduction to introduce the genes that will induce the pluripotent stage on somatic cells. It also utilizes feeder cells and medium containing xeno-products (Takahashi et al. 2007; Takahashi and Yamanaka 2006). Successful substitutions in reagents are being reported by a variety of authors: feeder cells are easily replaced by human extracellular matrix proteins, and culture media with serum are being replaced by serum-free media and recombinant molecules (Nakagawa et al. 2014; Stephenson et al. 2012; Wang et al. 2013). To avoid issues related to genotoxicity, especially insertional mutagenesis, non-integrative methods are

Table 2 Correction of iPSC from hematological diseases

Disease	Somatic cell for reprogramming	Reprogramming method	Known mutation(s)	Correction method	Differentiated cell	Reference
β-Thalassemia	Amniotic fluid cells	Episomal vector (Oct4, Sox2, Klf4, SV40LT, and miR-302–367)	C → T and 4-bp deletion (–TCTT) in *HBB*	TALEN	β-Globin expressing erythroblasts	Ma et al. (2013)
	Skin fibroblasts	Sendai virus (Oct4, Sox2, Klf4, and cMyc)	A → G and 4-bp deletion (–TCTT) in *HBB*	CRISPR/Cas9 and *piggyBac* transposon	β-Globin expressing CD34+ cells	Xie et al. (2014)
	Hematopoietic cells	Episomal vector	A → T mutation in *HBB*	CRISPR/Cas9	β-Globin expressing erythrocytes	Huang et al. (2015)
	Not described	Lentiviral vectors (Oct4, Sox2, Klf4, and cMyc)	A → T mutation in *HBB*	CRISPR/Cas9	β-Globin expressing CD34+ cells	Song et al. (2015)
	Not described	Sendai virus (Oct4, Sox2, Klf4, and cMyc)	4-bp deletion (–CTTT) in *HBB*	CRISPR/Cas9 and ssODN	β-Globin expressing erythrocytes	Niu et al. (2016)
Diamond-Blackfan anemia	Skin fibroblasts	Lentiviral vectors (Oct4, Sox2, Klf4, and cMyc)	Mutations in *RPS19* and *RPL5*	ZFN	Hematopoietic cells (hematopoiesis restored)	Garçon et al. (2013)
Dyskeratosis congenita	Skin fibroblasts	*Not described*	Mutations *Q31E*, *Δ37*, and *A353V* in *DKC1*	ZFN	*Not differentiated*	Gu et al. (2013)
Familial platelet disorder/ acute myeloid leukemia	Skin fibroblasts	Episomal vector (Oct3/4, Sox2, Klf4, L-Myc, and Lin28)	Mutations in *RUNX1*	ZFN	CD41+CD42+ megakaryocytes (megakaryopoiesis restored)	Connelly et al. (2014)
	Skin fibroblasts	Retroviral vectors (Oct3/4, Sox2, Klf4, and cMyc)	Mutations in *RUNX1*	TALEN	CD41a + megakaryocytes (megakaryopoiesis restored)	Iizuka et al. (2015)
Hemophilia A	Urinary epithelial cells	Episomal or Sendai virus (Oct4, Sox2, Klf4, and cMyc)	Chromosomal inversions in *F8*	CRISPR/Cas9	FVIII-producing endothelial cells	Park et al. (2015)
Severe congenital neutropenia	Peripheral blood mononuclear cells	Lentiviral vector (Oct4, Sox2, Klf4, and cMyc)	Mutations in *ELANE*	CRISPR/Cas9	Mature neutrophils (granulopoiesis restored)	Nayak et al. (2015)
Sickle cell disease	Skin fibroblasts	Lentiviral vector (Oct4, Sox2, Klf4, and cMyc)	E6V mutation in *HBB*	ZFN	*Not differentiated*	Sebastiano et al. (2011)
	Skin fibroblasts	Lentiviral vector (Oct4, Sox2, Klf4, and cMyc)	E6V mutation in *HBB*	TALEN and *piggyBac* transposon	*Not differentiated*	Sun and Zhao (2014)

recommended. Recombinant proteins, mRNA, microRNA, episomal vectors, piggyBac transposon system, or non-integrative viruses such as adenovirus and Sendai virus have been used as an alternative for reprogramming (Hong et al. 2013; Schlaeger et al. 2014). Various authors have reported episomal vectors as an alternative to retroviral transductions to reprogram peripheral blood mononuclear cells (PBMCs) to generate iPSC (Chou et al. 2011; Dowey et al. 2012; Liu et al. 2015). However, it has been reported that reprogramming efficiency is low when non-integrative methods, such as Sendai viral (0.1% efficiency for fibroblast reprogramming) and episomal methods (0.01%), are used. Despite the fact the RNA method shows higher efficiency (1.0%), compared to the others, generation of RNA-iPSC from blood cells seems to be unsuccessful (Schlaeger et al. 2014). Yet, these non-integrative methods show lower rates of aneuploidy and copy number variation compared to retroviral methods (Kang et al. 2015; Schlaeger et al. 2014). The episomal method presents low efficiency, however, because of the rapid loss of the reprogramming agent after few passages and the capability to be cultured in GMP compliant system, including xeno-free and feeder-free systems, this reprogramming method seems to be a suitable alternative (Goh et al. 2013; Schlaeger et al. 2014).

Some methodologies can be used to assess possible chromosomal abnormalities as a result of the reprogramming process in these cells. The choice of the method to be used will depend on the desired resolution. G-band karyotyping can detect gross chromosomal abnormalities. Taapken et al. (2011) reported that trisomy 12 is the most predominant karyotypic defect found in iPSC even when episomal vectors were the reprogramming method. Fluorescence in situ hybridization is another available method capable of detecting chromosomal translocations and aneuploidy. Also, molecular analysis such as single-nucleotide polymorphism arrays has higher resolution to monitor genetic aberrations (Hong et al. 2013; Kang et al. 2015). All the process from initial culture to transplantable cell products may contribute to risks for patients. Thus, it is essential to monitor and regulate these practices to ensure a safe and efficient therapy.

Obstacles to overcome in generating functional cells

Great variation exists among human iPSC lines regards their ability to differentiate specific strains. This variation is caused by different factors, i.e., residual DNA methylation. Even after reprogramming process, some iPSC remain with the epigenetic signature of their original cell, a phenomenon known as epigenetic memory (Nishizawa et al. 2016; Ramos-Mejía et al. 2012). Aberrations in DNA methylation occurs during the reprogramming process and leads to aberrant gene expression (Koyanagi-Aoi et al. 2013). Another factor is the genetic difference that already exists in the donors' cells. A recent study analyzed the hematopoietic differentiation capacity of 35 iPSC lines. They found that the gene IGF2 is responsible for the variation in hematopoietic commitment capacity among iPSC lines. Complex analyses, including gene expression, DNA methylation, and open chromatin accessibility, may help to predict the differentiation capacity of iPSC (Nishizawa et al. 2016).

Also, the generation of hematopoietic stem cell with long-term and self-renewal capability, as well as its differentiation in all blood cell types capable of effective oxygen transport, hemostasis, and innate and acquired immunity, remains elusive (Rowe et al. 2016). Cell-based therapy for hematological conditions requires many blood cell types, and researchers have reported their derivation: red blood cell (Dorn et al. 2015; Lapillonne et al. 2010), NK cell (Hermanson et al. 2016), megakaryocytes (Pineault et al. 2013), lymphocytes B and T (Kennedy et al. 2012; Liang et al. 2013), and others. However, these methods are not well developed for clinical purposes, and low efficiency of differentiation and quality of the cells produced are still an issue.

For a successful clinical application of pluripotent stem cell-derived cells, it is important that these cells survive in the recipient after transplant, integrate into desired tissues, and function properly. Researchers have reported poor bone marrow engraftment for hematopoietic stem cell transplants, approximately 0.1–2% of efficiency, when the cells were derived from ESC and iPSC (Liu et al. 2014). Abed et al. (2015) investigated the engraftment potential of non-human primate (NHP) iPSC-derived cells in NSG mice. They performed intra-femoral and retro-orbital injections of 10^6 unsorted cells. The cells injected into the right femur displayed up to 0.53% specific NHP-CD45+ cell engraftment. However, NHP cells were not detected in mice analyzed 12 weeks after transplantation. This indicates that engraftment capacity was transient. Conversely, the injection of 5×10^4 human cord blood CD34+ cells was sufficient to obtain up to 70% engraftment (Abed et al. 2015). To improve engraftment, Gori et al. (2015) investigated whether the presence of a

vascular niche that produces Notch ligand jagged-1 (JAG1) and delta-like ligand-4 (DLL4) drives definitive hematopoiesis. Findings indicated that endothelial Notch ligands promote definitive hematopoiesis and production of long-term engrafting CD34+ cells, suggesting that these ligands are critical for hematopoietic stem cell (HSC) emergence (Gori et al. 2015). Other strategies, such as the use of mesenchymal stem cells co-infused with hematopoietic stem cells (Fernández-García et al. 2015; Zhao and Liu 2016), NK cells (Escobedo-Cousin et al. 2015), and many others cell types, are being developed to improve cells engraftment. Also, ex vivo manipulation of HSC to enhance the responsiveness of HSC to BM-secreted chemoattractants and to promote HSC adhesion and seeding efficiency in the BM microenvironment has been reported (Briquet et al. 2010). However, more studies are needed to achieve a successful in vivo reconstitution of the hematopoietic cells.

Patent landscape for hematopoietic differentiation of iPSC

Since 2008, the total number of stem cell patents has declined. On the other hand, patents for iPSC technologies continue to increase (Mathews et al. 2013). There is no single company currently controlling the intellectual property (IP) for all techniques, methods, and reagents required for the production of iPSC (Rao 2013), but most of the patents filing deals with differentiation technologies (Roberts et al. 2014). We compare both landscapes of iPSC patents and patents dealing specifically with differentiation of iPSC into hematopoietic cells. To do so, we used a search query for iPSC patents (Roberts et al. 2014) in association with a search query for patents dealing with hematopoietic differentiation (coupled with several possibilities of the subject's occurrence[1]) in title, abstract, and claims of patents from 94 authorities provided by AcclaimIP (Anaqua Inc., Boston, MA) database of patents filed from 2006 to September, 2016. We found 2047 patent families (5089 patent documents) or patent inventions related to iPSC only, compared with 139 patent families (268 patent documents) related to hematopoietic differentiation of iPSC (HD + iPSC). The HD + iPSC patents showed a higher 5-year annual growth rate (51.76%) compared with the iPSC only group (36.46%). Despite fewer numbers of patents, this data shows that the IP of hematopoietic differentiation of iPSC has gained a great interest from R&D institutions. Both iPSC only and HD + iPSC patent groups have increased progressively in patent publications as shown in Fig. 4a. It is worth noting that in the last year, patent publication of HD + iPSC almost duplicated. The University of Kyoto, where the iPSC technology arose, remains as the leading player of iPSC-only patents with 95 patent families representing 4.4% from total, far from the second player which is the University of California, with 63 patents (2.3%). Cellular Dynamics International, a company from Fujifilm, comes as a leading player in HD + iPSC patents group, with twice as many inventions (n = 12) as University of Kyoto (n = 6), which occupies the second position in patent ownership (Fig. 4b). This leading position could be explained by the iPSC technology developed by Cellular Dynamics scientists named iCell® Hematopoietic Progenitor Cells, a cell-based system that can be differentiated into a variety of blood cells. The company has 30 iPSC patents, from those, 12 are related to HD + iPSC, whereas the University of Kyoto with 95 iPSC inventions has only five related to HD + iPSC. The top inventor of iPSC patents is Shinya Yamanaka (n = 45) (Takahashi and Yamanaka 2006). He serves as the director of Center for iPSC Research and Application and a professor at the University of Kyoto. He is also the second (n = 4) main inventor in HD + iPSC patents group. The leading inventor in this group is Amanda Mack (n = 5), a senior group leader at Cellular Dynamics International with scientific reports about the generation of iPSC from blood-derived cells (Mack et al. 2011) and iPSC differentiation into hematopoietic progenitors (Brown et al. 2010).

To understand the landscape of the patents and get insights of the inventions, we performed a matrix analysis of keywords occurrence within the patent

[1] Hematopoietic differentiation dataset search query: "TAC: (((haematopoietic ADJ different*) OR (hematopoietic ADJ cell* ADJ induc*) OR (differentia* NEAR7 hemocytoblast) OR ((differentia* NEAR7 (myeloid ADJ cell*)) OR ((differentia* NEAR7 (lymphoid ADJ cell*)) OR ((Produc* OR generat*) NEAR3 Hematopoietic ADJ Cell*)) OR ((Produc* OR Generat*) NEAR3 blood ADJ Cell*)) OR (hematopoietic ADJ cell* different*) OR (induc* NEAR4 (Hematopoietic ADJ cell*)) OR (Differenti* NEAR2 cell* NEAR2 blood) OR (Blood ADJ cell* ADJ different*) OR ((Different* OR Programm*) NEAR12 (hematopoietic ADJ cell*)) OR ((Different* OR Program*) NEAR12 (blood)) OR ((different* OR program*) NEAR12 (blood ADJ Cell*)) OR (Different* NEAR3 (bone ADJ marrow ADJ Cell*))))."

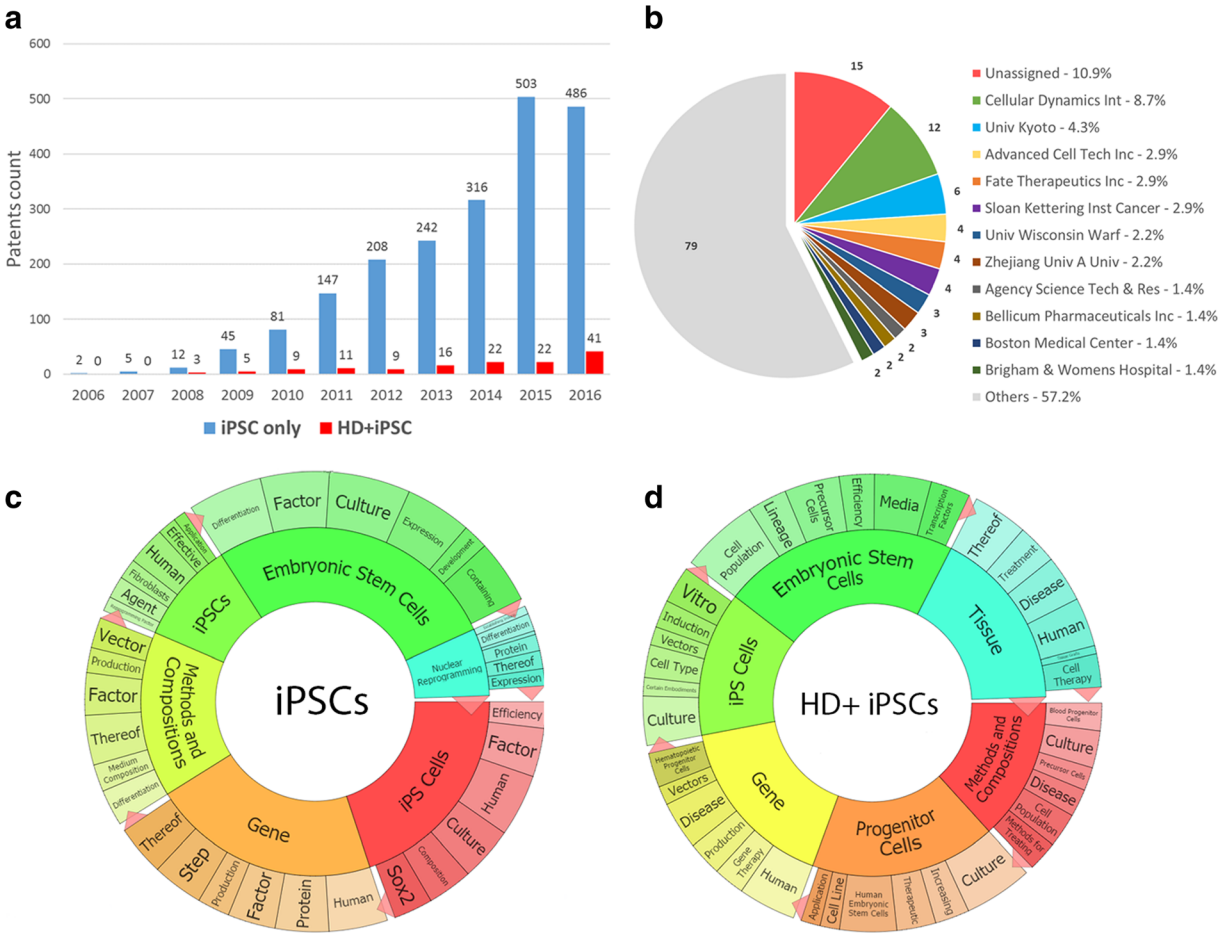

Fig. 4 Patent Landscape of iPSC and hematopoietic differentiation of iPSC (HD + iPSC). **a** Evolution of patent count by publication year of inventions related to iPSC and HD + iPSC. **b** Top 10 assignee of HD + iPSC patents including a group of unassigned patents. Conceptscape of 2-tier keyword association with all the patent documents related to *iPSC* (**c**) and *HD + iPSC* (**d**). The number of patents referring to each cluster is represented by the heat gradient (*red* means more patents in such cluster). Analysis performed from January 2006 to September 2016 (color figure online)

documents using a feature named Conceptscape, which clusters the keywords associated within the documents, in both groups. The results showed distinct landscapes regarding the words used to describe the inventions. Patents dealing with iPSC only have an exclusive "nuclear reprogramming" cluster and a higher frequency of terms "iPS cells" and "gene" within the documents than HD + iPSC group (Fig. 4c and d). Patents dealing with hematopoietic differentiation from iPSC have the cluster "methods and composition" as the term more frequently used in patent documents, which represents inventions more intended to describe the procedures to induce hematopoietic differentiation. The clusters "progenitor cells" and "tissue" were highly associated with HD + iPSC (Fig. 4d).

Pluripotent stem cells in clinical trials

In recent years, there has been a rapid increase in clinical trials comprising stem cell therapies. Numerous cell types, such as mobilized bone marrow cells, mesenchymal stem cells, adipose-derived stem cells, and umbilical cord blood cells have been used to target many diseases. However, a small number of these clinical studies involve PSC, for instance, hESC and iPSC (Table 3). The first trial using hESC-derived cells started in 2010, by Geron Inc. in the USA, intended to study the safety of human embryonic stem cell-derived oligodendrocyte progenitor cell targeting thoracic spinal cord injury. The technology was later acquired

Table 3 Selected pluripotent stem cell clinical trial

Clinical trial	Sponsor	Disease target	Cell	Phase	Start date	End date	Status
NCT01217008	Geron Inc. (USA)	Subacute thoracic spinal cord injury	hESC	I	October 2010	September 2013	Suspended
NCT01345006	Ocata Therapeutics (USA)	Stargardt's macular dystrophy	hESC	I/II	April 2011	August 2015	Ongoing
NCT01344993	Ocata Therapeutics (USA)	Dry age-related macular degeneration	hESC	I/II	April 2011	September 2015	Ongoing
NCT01469832	Astellas Institute for Regenerative Medicine (USA)	Stargardt's macular dystrophy	hESC	I/II	November 2011	September 2015	Completed
NCT02445612	Astellas Institute for Regenerative Medicine (USA)	Stargardt's macular dystrophy	hESC	I/II	July 2012	August 2029	Ongoing
NCT01625559	Chabiotech Co. Ltd. (S. Korea)	Stargardt's macular dystrophy	hESC	I	September 2012	June 2015	Unknown
NCT01674829	Chabiotech Co. Ltd. (S. Korea)	Dry age-related macular degeneration	hESC	I/II	September 2012	April 2016	Unknown
NCT02122159	Ocata Therapeutics (USA)	Myopic macular degeneration	hESC	I/II	March 2013	July 2016	Withdrawn
NCT02057900	Assistance Publique—Hôpitaux de Paris (France)	Heart failure	hESC	I	June 2013	June 2017	Active, recruiting
UMIN000011929	RIKEN (Japan)	Wet age-related macular degeneration	hiPSC	I	September 2014	–	No longer recruiting
NCT02239354	ViaCyte (USA)	Type I diabetes mellitus	hESC	I/II	September 2014	August 2017	Active, recruiting
NCT02302157	Asterias Biotherapeutics (USA)	Subacute thoracic spinal cord injury	hESC	I/II	March 2015	September 2018	Active, recruiting
NCT02286089	Cell Cure Neurosciences, Ltd. (Israel)	Dry age-related macular degeneration	hESC	I/II	April 2015	August 2017	Active, recruiting
NCT02749734	Southwest Hospital (China)	Macular degeneration diseases	hESC	I	May 2015	December 2017	Active, recruiting
NCT01691261	Pfizer (UK)	Wet age-related macular degeneration	hESC	I	June 2015	November 2016	Ongoing
NCT02590692	Regenerative Patch Technologies, LLC (USA)	Dry age-related macular degeneration	hESC	I/II	October 2015	September 2022	Active, recruiting
NCT02755428	Chinese Academy of Sciences (China)	Age-related macular degeneration	hESC	0	December 2015	December 2016	Active, recruiting
NCT02452723	Cyto Therapeutics Pty Limited (Australia)	Parkinson's disease	Human parthenogenetic-derived neural stem cells	I	March 2016	March 2019	Active, recruiting

by Asterias Biotherapeutics Inc. No serious safety issues occurred related to the cell transplants (Priest et al. 2015).

Most of the trials using pluripotent stem cell-derived cells are targeting eye degeneration disease. This is because the number of cells required for therapies is small, and the tissue is easily accessed for surgery and visualization of grafts. Furthermore, the differentiation of the pluripotent stem cell into the retinal epithelial cells is considered easy. However, there are other diseases being the target of stem cell therapies, such as heart failure, type I diabetes mellitus, and Parkinson's disease. Mobilized bone marrow cells and cells derived from cord blood are the most used in cell therapies for hematological diseases, such as sickle cell disease and beta thalassemia.

The first clinical trial using iPSC started in September 2014 by RIKEN in Japan. This study intended to evaluate the safety of iPSC-derived retinal pigmented epithelium (RPE) for the treatment of wet age-related macular degeneration (RIKEN-Foundation for Biomedical Research and Innovation 2013; University hospital Medical Information Network (UMIN) Center 2016). A single patient was treated; she received hiPSC-derived RPE generated from autologous fibroblasts. The group reported that the cells were non-tumorigenic, negative for viruses, and did not cause a graft versus host response. Even so, the treatment of a second patient was suspended in March 2015 due to a gene abnormality found in iPSC cells. They changed the strategy and will treat subsequent patients with partially matched donor cells already validated for genomic stability rather than autologous iPSC. This change was stimulated by new regulations for stem cell-based therapies that went into effect in Japan in November 2014 (Garber 2015).

Until November 2016, there were no clinical trials registered to ClinicalTrials.org employing iPSC or iPSC-derived cells for the treatment of hematologic diseases. In summary, it is too early to foresee any effectiveness of trials with pluripotent stem cells, but recent observations of patients indicate that they appear to be safe.

Conclusions

The first report of pluripotent human embryonic stem cell isolation (Thomson et al. 1998) created expectations for the use of these cells in clinical applications. Since then, efforts have constantly been made to generate cells with clinical grade. The development to reach these clinical grade cells includes the following: refinement of culture conditions, improvement in methods and tools to reduce or completely avoid the risk of transmitting pathogens, improvement in the differentiation techniques, and application of standards of quality assurance and good manufacturing practice (GMP) to satisfy regulatory aims of product (safety, quality, and efficacy).

The development of the technology of iPSC revolutionized the field of pluripotent stem cells. Issues related to ethics, immune rejection, and cell availability seemed to be solved. In the last 10 years, remarkable progress has been made to make possible the clinical utilization of iPSC. In the area of hematologic diseases, great progress was made in the development of in vitro differentiation, but the ultimate success in deriving bona fide HSCs for clinical applications remains uncertain. A greater understanding of molecular mechanisms underlying the generation of hematopoietic stem cells is necessary to produce transplantable hematopoietic stem progenitor cells from iPSC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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