Chapter 9
Gene and Cell Therapy for β-Thalassemia and Sickle Cell Disease with Induced Pluripotent Stem Cells (iPSCs): The Next Frontier

Eirini P. Papapetrou

Abstract In recent years, breakthroughs in human pluripotent stem cell (hPSC) research, namely cellular reprogramming and the emergence of sophisticated genetic engineering technologies, have opened new frontiers for cell and gene therapy. The prospect of using hPSCs, either autologous or histocompatible, as targets of genetic modification and their differentiated progeny as cell products for transplantation, presents a new paradigm of regenerative medicine of potential tremendous value for the treatment of blood disorders, including beta-thalassemia (BT) and sickle cell disease (SCD). Despite advances at a remarkable pace and great promise, many roadblocks remain before clinical translation can be realistically considered. Here we discuss the theoretical advantages of cell therapies utilizing hPSC derivatives, recent proof-of-principle studies and the main challenges towards realizing the potential of hPSC therapies in the clinic.

Keywords Induced pluripotent stem cells • Reprogramming • Cell therapy • Gene therapy • Beta-thalassemia • Sickle cell disease

Why Use iPSCs?

Gene therapy for beta-thalassemia (BT) using hematopoietic stem/progenitor cells (HSPCs) is already in the clinic [1, 2] and, although its efficacy and safety is still being evaluated, it has shown promising results, including clinical benefit in one patient [1]. One may therefore ask: why consider alternative strategies? We will first

E.P. Papapetrou (✉)
Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1044A, New York, NY 10029, USA
e-mail: eirini.papapetrou@mssm.edu

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discuss the challenges and limitations of current HSPC-based gene therapy for BT and sickle cell disease (SCD) and the potential benefits that iPSCs offer over HSPCs.

Limitations of HSPC-Based Gene Therapy

Current cell and gene therapy approaches for BT and SCD, similarly to all cell and gene therapy approaches for genetic disorders of the hematopoietic system more broadly, use HSPCs as the target cell type for harvesting, genetic manipulation and transplantation to the patient (Fig. 9.1). The overwhelming advantage of this strategy is that it exploits the remarkable ability of hematopoietic stem cells (HSCs) to reconstitute the entire hematopoietic system and capitalizes on extensive expertise gained by the field of HSC transplantation, pioneered by E. Donnall Thomas over half a century ago in Seattle. HSPC transplantation is the most widely utilized form of cell therapy today. A total of one million transplantations have been performed worldwide with more than 50,000 transplantations performed annually, based on World Health Organization (WHO) estimates, for malignant (primarily) and non-malignant disorders of the hematopoietic system.

The accumulated experience of the field has produced optimized protocols and standardized practices for the mobilization, harvesting, ex vivo manipulation, cryopreservation and administration of HSPCs to the patient. Adopting these practices,

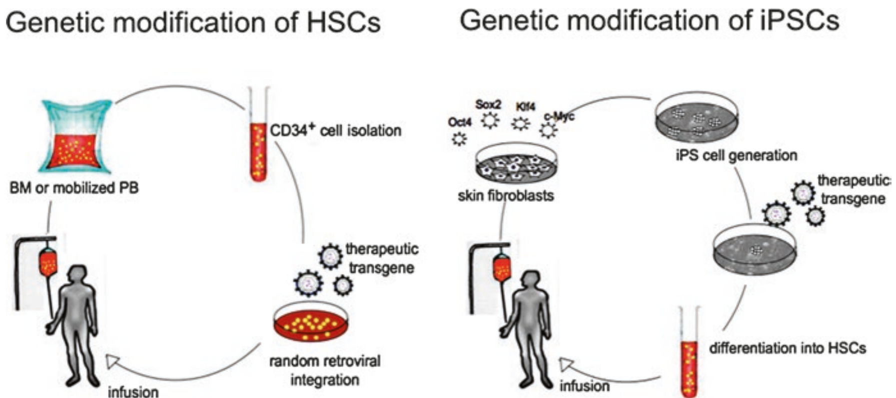

Fig. 9.1 Scheme of autologous cell and gene therapy for blood diseases using HSCs (*left*) or iPSCs (*right*). Left panel: The current paradigm of cell and gene therapy of the hematopoietic system entails harvesting of bone marrow or mobilized peripheral blood from the patient, purification of $CD34^+$ cells, transfer of the therapeutic transgene (through retroviral or lentiviral transduction), followed by infusion to the patient. Right panel: The future prospect of using iPSCs instead of HSCs would entail: isolation of somatic cells (skin fibroblasts are depicted in this scheme, but any other somatic cell type, such as blood cells, adipocytes, keratinocytes etc. could theoretically be used); reprogramming to derive one or more iPSC lines; genetic modification to correct the disease (here viral transduction is depicted, but any other strategy of genetic correction can be envisioned); differentiation into HSCs; and infusion to the patient

gene therapists can harvest hematopoietic cells, enrich them for HSPCs and, after a short period of culture, transfer the therapeutic gene and return the cells (some of which are now genetically modified) to the patient (Fig. 9.1).

Despite remarkable progress, some success and future promise, this practice suffers from an important limitation inherent to the use of HSPCs as the target cells. This is the inability to maintain HSPCs ex vivo for more than a short period of time (typically up to 72 h), since, despite extensive efforts, culture conditions permissive for the expansion or maintenance of HSPCs ex vivo at an undifferentiated state and without loss of their long-term engraftment ability, have yet to be found. The need to keep any ex vivo manipulation short-term imposes severe constraints to the genetic modification strategies: it restricts the choice of delivery methods, limits the possibility for "quality control" of the genetically modified cells and precludes sophistication, such as selection or exclusion steps. As a consequence, with these constraints, systems that allow highly efficient recovery of genetically modified cells in the bulk population and, specifically in the cell compartment with repopulating ability, are the only realistic option for a gene therapy regimen with therapeutic efficacy. Viral vectors derived initially from gamma-retroviruses (mainly murine leukemia viruses, MLVs) and subsequently lentiviruses (human immunodeficiency virus 1, HIV-1), have thus to date presented the only gene transfer technologies efficient enough to permit therapeutic levels of expression of the transgene in the reconstituted hematopoietic system. Efficient gene delivery by retroviral and lentiviral vectors hinges on random (or semi-random) integration in the genome, which is well-documented to mediate insertional mutagenesis. The latter entails the serious risk of promoting malignant transformation, most commonly by activation of expression by a vector-encoded promoter or enhancer and secondarily by disruption (leading to fusion or aberrantly spliced gene products) of cancer-promoting genes residing in the vicinity of the integration site. Insertional leukemogenesis constitutes the most severe and alerting shortcoming of HSPC gene therapy to date. Although this risk will likely be substantially reduced with more recent vector platforms (mainly taking advantage of self-inactivating, SIN, vector designs), possibly to levels acceptable for these therapies to become part of standard clinical practice, it will arguably never become negligible. Avoidance of random integration in HSPCs will perhaps become feasible with gene correction approaches using site-specific endonucleases, such as zinc finger nucleases, ZFNs, which have more recently been shown to be potentially efficient enough, at least for some gene therapy applications, but data on a human clinical setting are still pending. Even more importantly, the risk of transforming events in HSPCs mediated by off-target double stand breaks (DSBs) of the genome has not yet been adequately assessed and poses a real concern.

As will be discussed in the subsequent paragraph, the chief advantage of iPSCs as targets for combined gene and cell therapy is that genetic modification is performed in cells that can be maintained ex vivo and characterized extensively for the fidelity and precision of the genetic change, as well as for additional safety parameters, as needed (Table 9.1). The cells that pass this "quality control" and are deemed both adequate to mediate a therapeutic effect and safe can then be expanded to clinical scale and subsequently differentiated into the desired transplantable cell type.

Table 9.1 Comparison of characteristics of human HSCs and PSCs that impact on their use as targets for gene transfer and gene therapy

	HSCs	PSCs
Maintenance/expansion in culture	Very limited	Unlimited (but genetic alterations may arise)
Subcloning	Not feasible	Feasible
Reconstitution of hematopoietic system	Robust	Currently not feasible

iPSCs: Features and Origins

In a breakthrough study in 2006, Shinya Yamanaka at Kyoto University reported the derivation of cells with pluripotency characteristics directly from mouse somatic cells through the transient expression of only four genes, Oct4, Klf4, Sox2 and c-Myc [3]. A little over a year later, the same investigators, as well as the group of Jamie Thomson at the University of Wisconsin, independently, reproduced this in human cells [4, 5]. This amazing discovery—building on: previously discovered principles of reprogramming cell fate by Hal Weintraub (at the Fred Hutchinson Cancer Center in Seattle) [6], inducing pluripotency in somatic cells by John Gurdon (at the University of Oxford) in amphibia in the 50s, and Ian Wilmut (at the University of Edinburgh) in sheep in the 90s [7, 8], and technical advances in the isolation of human embryonic stem cells (hESCs) by Jamie Thomson, also in the 1990s [9]—opened for the first time the possibility of deriving patient-specific human pluripotent stem cells (hPSCs). Two unique characteristics of hPSCs make them extraordinary tools for both research and potential therapeutic applications: (a) the ability of unlimited self-renewal in vitro, providing the opportunity to maintain these cells indefinitely as cell lines, and (b) the potential for directed differentiation into all cell types that are found in the human body, at least theoretically. (Practically, the latter is contingent upon the availability of appropriate in vitro differentiation methods for a given cell type).

Due to these features, hPSCs (including iPSCs) offer opportunities for genetic manipulation that no other primary human cell type presents, critically dependent on the unlimited possibility for subcloning and expansion. This opens two unique possibilities: (1) inefficient but precise methods of genetic modification can realistically be applied, and/or (2) selection and “quality control” of rare single cells carrying a desirable (and no other) genetic modification is feasible.

Initial studies of iPSC generation used permanently integrated gamma-retroviral or lentiviral vectors to express the reprogramming factors (typically OCT4, SOX2, KLF4 and c-MYC, or other factor combinations), as these can mediate very efficient delivery and support factor expression at sufficient levels and duration required for successful reprogramming [10]. Despite the fact that the transgenic factors are typically profoundly silenced in established iPSC lines, their permanent integration in the genome of iPSCs raises a number of concerns: (a) even low levels of residual

factor expression may alter the molecular and possibly functional characteristics of iPSC lines [11]; (b) reactivation of the factors may inhibit differentiation or promote oncogenesis [12]; (c) it is well-known that random integration of retroviral vectors causes insertional mutagenesis.

The observation that factor expression is not required beyond the end of the reprogramming process—as established mouse and human iPSCs were shown to have silenced the transgenic factors and to have activated endogenous pluripotency genes [4, 10, 12, 13]—and the need to move the field towards more clinically relevant methodologies of reprogramming drove several investigators to independently explore techniques for iPSC generation devoid of permanent factor integration. The first generation of vectors circumventing permanent factor integration were excisable vectors, which are effectively vectors—either plasmid or lentiviral—which initially integrate in the genome, but can be subsequently removed, after completion of reprogramming in established iPSC lines when expression of the factors is no longer needed (and in fact is most often already profoundly silenced). Excisable systems that were developed include plasmids [14] or lentiviral vectors [11, 15–19] excisable through the Cre/loxP or the piggyBac transposon/transposase system [14, 20, 21]. The main advantage of excisable systems, especially lentiviral, is that they maintain the very high reprogramming efficiency of integrating vectors. One disadvantage is the requirement of an additional step of transiently exogenously expressing the recombinase (or transposase) and selecting clones with documented complete excision—which extends the passaging time in culture. Another notable disadvantage is that Cre-excisable vectors leave behind a loxP site (~30 nucleotides) as a "footprint" and care should be taken to ensure this does not impact the function of the cell's genome in any way. We proposed that a residual loxP site can be considered practically "harmless" if it is outside coding sequences [22]. By this rule, the vast majority of residual loxP sites in iPSCs should be inconsequential. Alternatively, transposase systems can mediate "seamless" excision, but the possibility of transposition to another genomic site should be excluded.

Subsequently, methods to derive transgene-free mouse or human iPSCs that are completely devoid of integration were developed [23]. These include (a) non-integrating DNA vectors, (b) DNA-free methods. The first category includes adenoviral vectors [24], conventional plasmids [25], oriP/EBNA1 episomes [26] and minicircles [27]. Although all these methods have the potential to generate genetically unmodified iPSC lines, their main disadvantage is the generally low efficiency of reprogramming, which is often several orders of magnitude below this of integrating vectors and therefore often insufficient for derivation of patient-specific iPSC lines from primary biopsies of adult patients. A second concern is that iPSCs derived with these methods still need to be tested for lack of integrated DNA and may harbor randomly integrated vector fragments that may escape detection by standard PCR-based techniques. DNA-free methods are based on RNA-mediated delivery - by Sendai virus-based vectors [28, 29] or repeated transfection of modified mRNAs [30] - or protein delivery using recombinant proteins or cell extracts [31–33]. The main advantage of these methods is the derivation of iPSC lines totally free

of DNA integration. While the extremely low efficiency and reproducibility of reprogramming by protein delivery renders the latter an unrealistic option at this time, RNA delivery offers adequate reprogramming efficiency.

Production and repeated transfection of modified RNAs is rather cumbersome and not suitable for many cell types other than fibroblasts, particularly non-adherent ones, such as cells of hematopoietic origin. Commercialization of Sendai viruses expressing the four "Yamanaka" reprogramming factors has made this technology broadly accessible and increasingly popular. Finally, ongoing efforts are directed towards developing reprogramming methods not requiring any genetic manipulation of cells through the use of small molecules [34] .

Proof-of-Principle Studies Using iPSCs for Gene and Cell Therapy of BT and SCD

The hemoglobinopathies are the most extensively studied inherited monogenic disorders in the human population and their correction by gene therapy has inspired generations of investigators over several decades. It was therefore no surprise that SCD was selected for the first ever proof-of-principle study of gene and cell therapy with autologous iPSCs. Jacob Hanna, at the time a post-doc in Rudolf Jaenisch's lab, performed a tour-de-force study in the mouse, published in a seminal Science paper only 16 months after the original Yamanaka study [36]. Using a humanized mouse model of SCD developed in Tim Townes' laboratory [37], the Hanna et al. study provided first proof of principle of a general scheme of autologous iPSC-based cell and gene therapy, which includes the following steps: (1) reprogramming of somatic cells (specifically, tail-tip fibroblasts) into iPSCs, (2) genetic correction (by "classic" homologous recombination), (3) in vitro differentiation into hematopoietic progenitors and (4) transplantation. Although this study provided clear proof-of-concept early on in the reprogramming field, many aspects of it render it not directly translatable: permanently integrated retroviruses expressing three of the reprogramming factors were used for reprogramming, the iPSC clones were only minimally tested for genetic aberrations and, perhaps most importantly, long-term multi-lineage reconstitution of the hematopoietic system was not shown.

Since the first generation of human iPSCs, a few groups have generated iPSCs from patients with SCD [38–42] and BT major of various β^0/β^0 or β^0/β^+ genotypes [22, 43–46]. Different starting cell types, including skin fibroblasts [22, 38, 41–44], bone marrow fibroblasts (or mesenchymal stem cells, MSCs) [22, 39], amniotic fluid cells [45, 46] peripheral blood mononuclear cells [40] and a variety of reprogramming methods, including retroviral vectors [22, 42–44], a single excisable lentiviral vector [22, 41, 45], EBNA1/oriP-based episomes [40, 42, 46] and a piggyBac transposon vector [39] have been used, reflecting the advances of reprogramming methods in the iPSC field along this period.

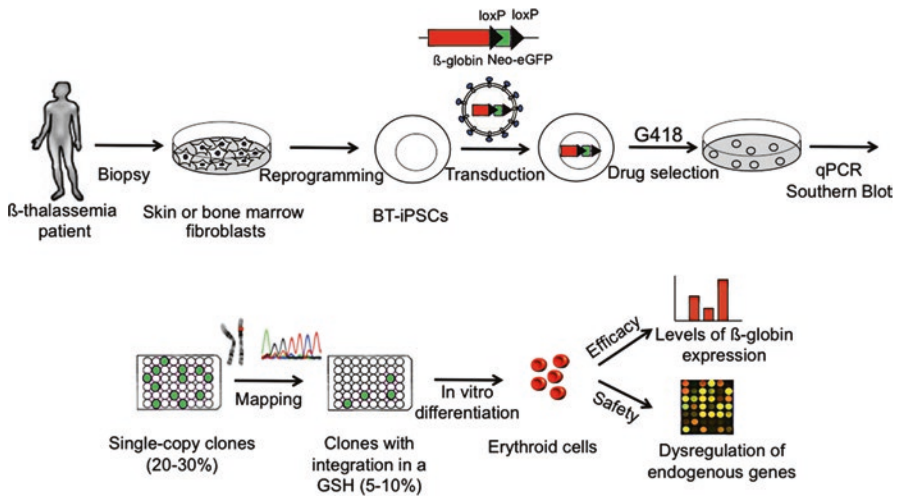

Fig. 9.2 Genetic correction of BT-iPSCs by insertion of a β-globin gene into genomic safe harbors. iPSCs generated from BT patients are transduced with a lentiviral vector driving erythroid cell-specific expression of the β-globin gene. Clones with single integrations can be identified (initially by qPCR and subsequently by Southern Blot) and comprise approximately 20–30% of all G418-resistant clones when transduction is performed at an optimal MOI (0.1–0.3). 1 out of 10 to 20 of these contains integrations at sites that meet the five safe harbor criteria by Papapetrou et al. To further test efficacy and safety in vitro, erythroid cells can be derived and gene expression assays performed to assess β-globin expression and expression of genes in the vicinity of the vector integration (see also text for details)

The first genetic correction study in a human setting was provided by Papapetrou et al. from the Sadelain lab [22] (Fig. 9.2). This study further proposed a new strategy for genetic correction, using gene addition as opposed to in situ correction, followed by selection of corrected clones harboring single copies of a normal beta-globin gene allele inserted into "safe harbor" sites in the human genome. Specifically, we derived multiple BT-iPSC lines from BM fibroblasts from four patients of various genotypes (β^+/β^0 and β^0/β^0). We developed a lentiviral vector derived from the TNS9 vector originally developed at the Sadelain laboratory [47] and similar to the various lentiviral vectors currently tested in preclinical studies and clinical trials. This vector encodes the human β-globin gene driven by its autologous promoter under control of the locus control region (LCR) elements DNAse I hypersensitive sites HS2, HS3 and HS4 and is modified to also express a floxed PGK-Neo cassette for selection. The strategy developed was as follows: The vector was transduced into BT-iPSC lines at low multiplicity of infection (MOI), so that clones with single vector integrations could be isolated after single-cell subcloning and G418 selection. Clones that were found to harbor a single vector copy and thoroughly confirmed to be clonal were selected for mapping of the vector integration. Integration sites were tested against a set of criteria to select for sites that are less likely to perturb endogenous gene function. The proposed "safe harbor criteria" were

intended to avoid the two types of insertional events that predominantly result in gene dysregulation: transactivation of adjacent genes by a promoter/enhancer present in the vector (which is the most frequent mechanism of insertional mutagenesis/oncogenesis) and gene disruption. Five criteria were thus proposed to exclude regions of the genome in close proximity to coding and non-coding genes—requiring extra distance from genes known to play a role in cancer (in humans or model organisms) and conserved genetic elements. They also excluded integrations inside transcription units. Differentiation along the erythroid lineage showed that vector-encoded β-globin expression can reach therapeutically relevant levels in the majority of clones, including clones with safe harbor integrations. Finally, gene expression analysis was used as an additional safeguard step to ensure that no perturbation of endogenous genes is caused by the therapeutic vector. This is a potentially clinically translatable and universal approach to autologous cell and gene therapy for BT. Aspects warranting further investigation are discussed in the next section.

An alternative and more precise strategy for genetic correction is exploiting homologous recombination to repair the mutation in the endogenous gene locus, a strategy also referred to as homology-mediated gene repair. This strategy takes advantage of the cell's endogenous DNA repair machinery, activated in response to DNA double strand breaks (DSBs), and uses gene targeting tools and principles that have been developed for transgenesis in the mouse over the past two decades [48, 49]. Although conceptually simple, "in situ" genetic correction in human cells, including hPSCs has proven much more challenging than in mESCs. Although the reasons for this are not well understood, different properties of human and mouse PSCs, both biological and culture-related, namely the different growth properties and ability for single-cell subcloning, have been implicated. More recent improvements have made gene targeting in hPSCs feasible and relatively efficient. These include: (a) advances in cell culture conditions, enabling feeder-free single-cell survival of hPSCs (i.e. use of matrices and the Rock inhibitor Y-27632 [50], respectively), (b) improvements of gene delivery methods (i.e. optimized transfection/nucleofection conditions and viral systems for hPSCs) and (c) engineering of designer site-specific endonucleases, which can substantially boost the efficiency of HR [51]. The latter include zinc-finger nucleases [52], meganucleases [53], transcription activator-like effector (TALE) nucleases [54] and more recently the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas9 system [55].

Five studies in the past 3 years reported homologous recombination-based strategies for the genetic correction of mutations in iPSCs from patients with SCD [41, 42, 56] and BT [44, 46] (Table 9.2). One study used "classic" HR [44], one helper-dependent adenoviral vector (HDAdV)-mediated HR [42] and the other three HR facilitated by engineered endonucleases, either ZNF [41, 56] or TALENs [46]. Various donor constructs were used, delivered by electroporation (except in Li et al who used HDAdV) with or without plasmids encoding the nuclease (Fig. 9.3). The selection cassette was inserted in these studies either in the first intron, the second

Table 9.2 Genetic correction strategies in human BT- and SCD- iPSCs

Disease	Genotype	Correction method	Correction efficiency[a]	Beta-globin expression	Refs.
BT	β39/IVS1-110 (β^0/β^+) β39/IVS1-1 (β^0/β^+) β39/β39 (β^0/β^0)	Lentivirally-mediated gene addition in safe harbor sites	5–10% (clones with insertions in safe harbor sites)	Therapeutic levels in vitro (85% of normal allele)	Papapetrou et al.
SCD	βE6V/βE6V	ZFN-mediated HR	1 out of 300 resistant clones	25–40% of normal allele	Zou et al.
SCD	βE6V/IVS-1 βE6V/βE6V	ZFN-mediated HR	Between 5% and 38% depending on ZFN pair (multiple ZFN pairs tested)	Not assessed	Sebastiano et al.
SCD	βE6V/βE6V	HDAdV-mediated HR	81% (39 out of 41 clones)	Not assessed	Li et al.
BT	β41/42/β41/42 (β^0/β^0)	"Classic" HR	0.81% (2 out of 248 clones)	Detected in vivo (in SCID mice) short-term, not quantified	Wang et al.
BT	IVS2-654/IVS2-654 (β^0/β^0) β41/42/β41/42 (β^0/β^0)	TALEN-mediated HR	68% for IVS2-654 (25 out of 37 clones), 40% for β41/42 (4 out of 10 clones)	Detected in vitro, not quantified	Ma et al.

HR homologous recombination; *ZFN* zinc-finger nuclease; *HDAdV* helper-dependent adenoviral vector; *TALEN* Transcription activator-like effector nuclease
[a]Correction efficiency is given as ratio of corrected clones over drug-resistant clones

intron or downstream of the gene. The latter positioning seems preferable to avoid disrupting gene function. Indeed in the Zou et al. study by Linzhao Cheng's group, expression of the corrected β-globin allele after insertion of the selection cassette in the first intron was lower than normal and increased after Cre excision to only reach levels of about 25–40% of these of a normal allele. Even though other reasons (a mutation in the GATA binding site of the 3′ enhancer introduced during or after HR) could not be excluded, this result would warrant caution against targeting a selection cassette in the first intron. The donor DNA design used in Li et al. and Ma et al. containing the entire β-globin gene in the 5′ homology arm is suitable for correction of the majority of BT mutations and deletions (albeit conceivably with varying efficiencies). Expression from the corrected β-globin gene allele was unfortunately

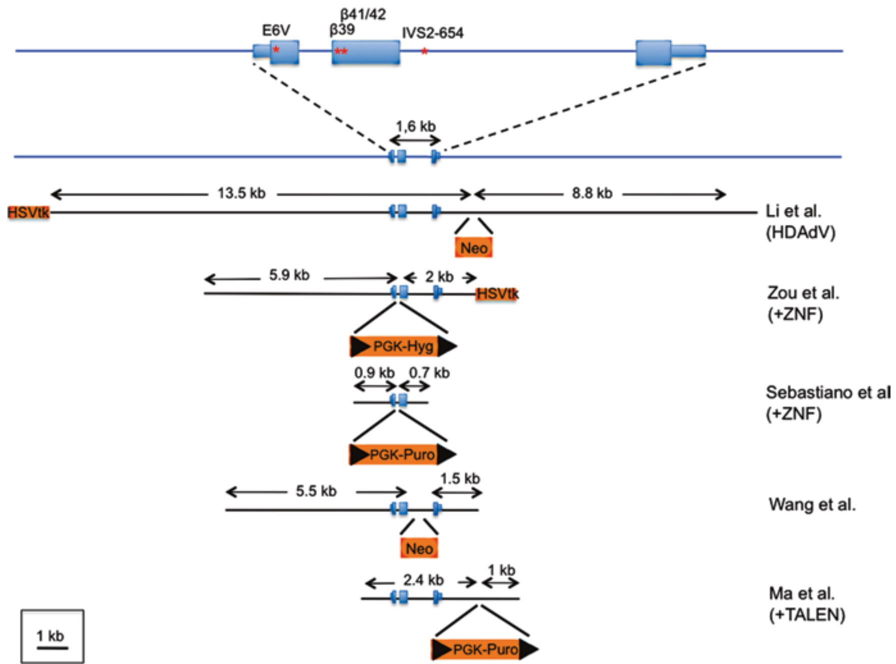

Fig. 9.3 Genetic correction of mutations in BT- and SCD- iPSCs by homologous recombination-based strategies. *Upper panel*: The β-globin gene locus with the BT- and SCD- mutations corrected in patient-derived iPSCs shown with asterisks (see also Table 9.2). The entire human β-globin gene spans approximately 1.6 kb on chromosome 11 and consists of 3 exons. Wide boxes depict coding regions, narrow boxes depict the 5′ and 3′ untranslated regions (UTRs). SCD is caused by a point mutation in codon 6 (E6V). The β39 is a C→T (CAG-TAG) nonsense mutation in codon 39 (at the beginning of exon 2) and is the most common β^0 gene mutation in Italy, mainly Sardenia. β41/42 (-TCTT) is a 4-bp deletion at codons 41/42 resulting in frameshift that generates a downstream stop codon, also leading to absence of β-globin gene expression (β^0). It is the most common BT mutation in China and Thailand. IVS1-1, IVS1-110 and IVS2-654 are single-base substitutions resulting in aberrant splicing of the first or second intron, common in Italy (IVS1-1, IVS1-110) and China (IVS2-654). *Lower panel*: scheme of targeting vectors used in five studies, as shown, for HR-mediated correction of BT- or SCD- iPSCs. Notable differences in the length of the homology arms and in the position of insertion of the selection cassette are depicted (see text for details)

not quantified in the other studies, although it was detectable in Ma et al. and Wang et al., in vitro and in vivo in SCID mice [44].

Although restoration of expression of a targeted allele to normal levels has yet to be demonstrated and the optimal design of the targeting vector has yet to be defined, these recent studies provide proof-of-principle that the β-globin gene locus can be targeted in hPSCs. Remaining roadblocks to clinical translation are further discussed below.

Promises and Challenges of iPSCs for the Gene and Cell Therapy of BT and SCD

Despite the promise of iPSCs in delivering cell and gene therapies for BT and SCD, several roadblocks on the road to clinical translation remain to be addressed.

Issues Common to All Applications of iPSC-Based Cell Therapy

Generation and Quality Control of iPSC Lines

First off, iPSC lines intended for clinical use will need to be generated using methods and procedures that meet both scientific and regulatory standards.

The main issues that need to be determined are: what is the preferred reprogramming method; what is the best starting cell type; what quality control is necessary to deem an iPSC line acceptable for clinical use. Regarding reprogramming methods, DNA-free RNA-based genetic methods seem currently the most appealing, as they combine safety with reasonable efficiency. As additional new methods—substituting some or all reprogramming genes with chemicals, or other treatments—will likely emerge, it is also important to keep in mind that adequate efficiency is required, not only to ensure time- and cost-efficient derivation of sufficiently numbers of iPSC lines to cover all needs, including derivation of lines from rare donors, but also—perhaps more importantly—to avoid selection of rare cells with extreme reprogramming fitness present in the starting cell population. Several connections between reprogramming fitness and transformation potential exist, raising the concern that such cells may be more likely to give rise to a malignant cell clone by acquiring additional genetic lesions before or after transplantation. Indeed, it is being increasingly appreciated that the degree of genetic mosaicism in somatic cells is substantial and that somatic genetic variants can have strong positive or negative effects on the cell's reprogramming ability [57–64].

This consideration also has implications for the choice of the most appropriate starting cell type. The ideal somatic cell to derive iPSCs from should be easily accessible (for example skin or blood), easily reprogrammable and less likely to harbor pre-existing genetic alterations. Studies combining reprogramming with high-throughput genomics will be needed to determine the degree of somatic mosaicism in different human tissues and to catalogue variants with an impact on reprogramming efficiency which may drive or predispose to cancer. Even less clear are standards needed to deem an iPSC line genetically "normal". Several studies have shown that iPSC lines often harbor genetic aberrations, in the form of chromosomal abnormalities, as well as more subtle copy number variants (CNVs) and single-nucleotide variants (SNVs) [65–72], most if not all of which pre-exist in the starting cell [73, 74]. Accumulating genomic data from large projects, such as the 1000 Genomes Project, The Cancer Genome Atlas project and others, will increasingly

over the next years lead to a better classification of genetic variants that confer risk of cancer or of non-malignant diseases. iPSC lines intended for cell therapies will likely need to be tested with high-resolution techniques, like whole-genome sequencing. Additionally, iPSC lines intended for use in cell therapies will need to be compatible with good manufacturing practice (GMP) standards and eligible for FDA approval (and therefore need to meet the FDA's tissue donor standards regarding donor selection, consent and screening) [75]. Finally, advances in the manufacturing procedures, including automated processing and large-scale culture in bioreactors will be required.

Autologous vs Histocompatible iPSCs

The generation of new iPSC lines is very time-consuming and laborious, rendering the prospect of autologous iPSC cell therapy, at least by today's standards, prohibitively expensive and hence unlikely to become routine medical practice. An alternative and more realistic solution would be the generation of cell banks of few allogeneic clinical GMP lines that are compatible for the majority of the population [76, 77]. At least partial human leukocyte antigen (HLA) matching will be required. This requirement may be more stringent in the case of HSC transplantation compared to transplantation of other tissues, according to the paradigm of solid organ transplantation. An attractive possibility is the generation of cell lines from donors homozygous for common HLA haplotypes.

It is estimated that 78% of Northern Europeans, 63% of Asians, 52% of Hispanics and 45% of African Americans would have a matched line if 100 HLA homozygous cell lines from each of these populations were generated, following extensive screening of hundreds of thousands of individuals [78].

Another possibility is the genetic engineering of "universal donor" iPSC lines to generate either HLA-homozygous or HLA class I- and/or class II-negative cell lines, for example by biallelic disruption of the beta-2 microglobulin gene, an obligatory component of HLA class I antigens [79].

All these different options will need to be informed by future studies addressing the immunogenicity of autologous or HLA-matched iPSCs and of their products and their susceptibility to immune rejection, issues that remain at present incompletely understood [80–83]. The antigenicity conferred by the expression of neoantigens by in vitro generated cells is also a matter warranting further investigation. Several open questions remain that include the degree of HLA matching required (which will likely depend on the derivative cell type), what is the best strategy to derive donor cell banks and whether concomitant immunosuppression will be required. Cell banks seem a more attractive option, especially in regions with relatively low ethnic and racial diversity (e.g. Japan). An "off-the-shelf" therapy combined with some level of immunosuppression seems at present the most likely scenario for broad applications of this technology.

Teratoma Formation and Tumorigenicity

A major concern of the transplantation of hPSC-derived cell products is the potential for tumor formation. At least three scenarios can be imagined that pose risks. First, residual pluripotent cells that resist differentiation may persist in a cell graft and give rise to teratomas, non-malignant tumors consisting of tissues of more than one embryonic germ layer, upon transplantation [84–87]. Second, partially differentiated progenitor cells may be present in the graft and result in aberrant proliferation. Even if these cell growths are not malignant, they can cause problems, especially if localized in regions of the body such as the central nervous system or the myocardium. Third, terminally differentiated cells may undergo de-differentiation and malignant transformation. There is some limited evidence that iPSC-derived cells may possess increased ability for transformation. This property may be due to accumulated genetic lesions that predispose to cancer or to unstable epigenetic marks that enhance de-differentiation and/or transformation. The tumorigenic tendency may vary depending on the cell type of origin of the iPSC line [88]. Although it is conceivable that improved differentiation protocols can minimize the chance of the first two scenarios, some method of additional purging of residual undifferentiated cells [89] and/or positive selection of differentiated progeny [90–92] may be beneficial at least in some applications. Furthermore, genetic safety switches can be engineered in the cell lines, for example exploiting suicide genes [93, 94].

Issues Common to iPSC-Based Cell Therapy of the Hematopoietic System

Generation of HSCs with Long-term Engraftment Potential

Two general culture systems are currently employed to induce hematopoietic differentiation of hPSCs: co-culture on stromal cells, typically the OP9 murine bone marrow stroma line, and formation of so-called embryoid bodies (EBs), i.e. aggregates of cells that are forced to stay in suspension, a process that triggers spontaneous differentiation. Although hematopoietic progenitors and more differentiated cells of all hematopoietic lineages can be derived from hPSCs, robust derivation of HSCs—defined by the ability for long-term engraftment and differentiation into all hematopoietic lineages—has not been possible so far.

The current inability to differentiate hPSCs (iPSCs and ESCs) into engraftable HSCs constitutes perhaps the predominant roadblock to cell therapy for blood disorders [95, 96]. Indeed the generation of HSCs from hPSCs has proved much more challenging than anticipated and is still elusive despite substantial efforts from a number of laboratories. Studies from different groups have invariably achieved very low (typically less than 2%) or no engraftment in mice and mostly restricted to the myeloid lineage [97–101]. Intrafemoral injection and transplantation into newborn NSG mice did not result in substantial improvement. Although reproducible and

efficient in vitro derivation of HSCs from hPSCs remains elusive despite intense research efforts, it is clear that this constitutes a technical limitation and that hPSCs inherently possess the biologic potential to give rise to HSCs [102, 103]. A number of reasons may impede success. Current xenograft models may impose "artificial" requirements for the long-term engraftment of hPSC-derived HSCs. Additional signals provided by components of the niche, which may be needed for specification or engraftment, may be critically missing. hPSC-derived hematopoietic cells, similarly to other cell types derived from them, seem to have a more embryonic-like or primitive-like developmental phenotype. Yolk-sac derived hematopoietic progenitors inherently lack the ability to engraft adult recipients and several studies have shown that at least a proportion of hematopoietic cells derived from hPSCs cultures (typically an early "wave") resembles this type of progenitors, although these cultures have been convincingly shown to also give rise to definitive-type hematopoietic cells. Finally, insufficient knowledge of the appropriate culture conditions limits the ability to "capture", maintain and expand HSCs, which may only transiently emerge in these cultures. Insights into the ontogeny of the mammalian hematopoietic system from the developmental hematopoiesis field [104] and/or "trial-and-error" testing of culture conditions, growth factors and cell-to-cell signals by the stem cell field, will likely eventually make the long sought-for goal of HSC generation from hPSCs a reality.

Strategies for Genetic Correction

As discussed above, iPSCs open many more possibilities for sophisticated genetic manipulation than HSCs. Two main approaches for genetic correction discussed above include gene addition in safe harbor sites and HR-mediated gene repair. Gene addition in safe harbors provides a potentially universal approach to disease correction for many diseases caused by reduced or absent expression of a gene and allows the simultaneous expression of supernumerary genes, such as suicide or drug selection genes. A scheme like the one proposed in Papapetrou et al. (Fig. 9.2), where clones with random integrations are screened prospectively or, alternatively, gene targeting of a pre-selected safe harbor site, can be envisioned. However, although the safe harbor criteria provide a starting point, additional data will be needed to establish universal safe harbor sites. Ongoing annotation of the human genome and better understanding of its function can help refine current criteria, for example by incorporating information on long-range chromatin interactions, non-coding RNAs and regulatory DNA.

Editing of the endogenous β-globin locus is also an attractive approach. Although the β-globin gene is silent in hPSCs, and thus its targeting challenging [105], several studies—discussed above—have now demonstrated feasibility. BT is caused by a very large number of different mutations spanning the entire gene, but the β-globin gene comprises only 3 small exons and 2 small introns spanning approximately 1.6 kb. Therefore, the development of a targeting correction vector appropriate for most mutations is conceivable, even though the efficiency would vary between patients

with different genotypes and with potential polymorphisms in the homology regions. Monoallelic correction would be sufficient for phenotypic correction, provided that the corrected locus is expressed at the levels of a normal allele. A considerable body of work involving systematic comparisons of different donor vector designs with or without DSB induction by endonucleases and different strategies for delivery of the donor DNA will be needed, as well as additional data on the efficacy and safety of different nucleases from different families and with different site specificity. ZFNs are ahead of other nuclease technologies in establishing a clinical record in cell therapies [106], but targeting stem cells rather than more differentiated cell types imposes a much higher burden of proof of safety. In parallel, assays of endonuclease-induced genotoxicity, primarily due to off-target cleavage, will need to be established and standardized and acceptable thresholds will need to be defined and amended, as more data become available.

Since iPSCs are clonable and expandable cells, lower efficiency of gene targeting can be afforded and traded for enhanced safety, as obtaining only one or a few corrected clones is sufficient. Regardless of the strategy of genetic correction, it is important that extended culture be avoided, as this will increase the frequency of acquisition of genetic abnormalities [66]. Genetic manipulation will still almost unavoidably necessitate prolonged passaging of the iPSC lines. Thus, standards for verifying genomic integrity, as discussed in the previous section, will be essential.

Issues Specific to iPSC-Based Cell Therapy for BT and SCD

Transfusion Products from iPSCs

Many patients with BT and SCD would also benefit from the development of transfusion products from iPSCs. Although this would not provide long-term treatment, autologous or compatible red blood cells (RBCs) could be valuable for patients with antibody sensitization, often occurring in these patients during multiple transfusions. Either erythroblasts or mature RBCs could be used to this end. The latter would provide a shorter-term treatment and require more prolonged in vitro manufacturing, likely raising the associated cost. On the other hand, the possibility to irradiate the RBC product before transfusion could eliminate the major safety concerns associated with stem cell therapies. Indeed, infusions of RBCs derived from hPSCs represent one of the most appealing near-term strategies for iPSC-based therapies. Although RBCs do not express HLA, they do express a number of surface antigens. Antibody formation against almost 400 antigens belonging to thirty families (blood group systems) has been described [107]. This makes it hard to envision iPSC banks matching all combinations, but the possibility of a master bank of only a few lines generated by donors negative for Rhesus and for as many as possible of the minor antigens is conceivable.

Several studies have demonstrated the feasibility of generating erythroid cells from human ESCs and iPSCs in vitro using different protocols [22, 108–110]. It is clear from these studies that hPSC-derived erythroid cells express predominantly embryonic (ε) and fetal (γ) globins and practically no β-globin. Although protocols

to induce the globin switch in vitro may be developed in the future, an intriguing idea is to harness this developmental immaturity of hPSC-derived erythroid cells to derive phenotypically "corrected" cells from SCD and BT patients without genetic engineering.

Studies assessing the functionality and immunogenicity of in vitro generated RBCs, as well as advances in cell manufacturing, will be needed before protocols for cost-efficient, routine, large-scale production of RBCs can be implemented [111]. An intriguing possibility is the derivation of self-propagating erythroid progenitor lines capable of induced terminal differentiation from iPSCs that can be expanded and banked at the progenitor stage, thus reducing variability, time and cost [107].

Concluding Remarks

The iPSC technology has received a lot of attention from the scientific community and the media alike and holds great promise for the development of new treatment options for BT, SCD and dozens of other inherited and acquired hematopoietic disorders. Some of its applications in regenerative medicine, like the generation of blood transfusion products, seem imminent. Others, on the other hand, like the derivation of transplantable HSCs, seem further away. Advances in several fronts, such as the derivation of high-quality iPSC lines, the development of better differentiation protocols, standards for quality assurance, regulatory frameworks and cost-efficient manufacturing procedures, will be essential for moving this exciting new technology from proof of concept to the clinic.

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