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REVIEW ARTICLE

Gene therapy and genome editing for primary immunodeficiency diseases

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Abstract In past two decades the gene therapy using genetic modified autologous hematopoietic stem cells (HSCs) transduced with the viral vector has become a promising alternative option for treating primary immunodeficiency diseases (PIDs). Despite of some pitfalls at early stage clinical trials, the field of gene therapy has advanced significantly in the last decade with improvements in viral vector safety, preparatory regime for manufacturing high quality virus, automated CD34 cell purification. Hence, the overall outcome from the clinical trials for the different PIDs has been very encouraging. In addition to the viral vector based gene therapy, the recent fast moving forward developments in genome editing using engineered nucleases in HSCs has provided a new promising platform for the treatment of PIDs. This review provides an overall outcome and progress in gene therapy clinical trials for SCID-X, ADA-SCID, WAS, X- CGD, and the recent developments in genome editing technology applied in HSCs for developing potential therapy, particular in the key studies for PIDs.

Introduction

PIDs comprise of a large more than 300 gene mutations consisting of heterogeneous group of rare heritable disorders resulting in functionally compromised immune system or underdeveloped severe immuno deficiencies

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with high mortality. Although allogeneic hematopoietic stem cell transplantation (HSCT) approach can cure PIDs, the majority of patients are not accessible for this treatment due to lack of the HLA matched donor. The patients underwent HSCT with less matched donor often suffer from graft-versus-host (GVHD) disease resulting in delayed immunological reconstitution and a significant problem of graft rejection.[1] This has led to develop an alternative gene therapy based on the transplantation of genetically corrected autologous HSCs using the modified viral vector. The clinical therapeutic benefit via autologous gene transfer was first demonstrated in the X-linked severe combined immunodeficiency (X-SCID) patients.[2] Since then, this type approach continues to exhibit promise with clinical applications, including other PIDs in last two decades. Development of a safe and efficient viral vector has been one of major progress in gene therapy field in the past. The issue brought to light due to the viral enhancer mediated mutagenesis observed in the early gene therapy trial patients.[3] This led to develop the self-inactivating (SIN)gamma-retroviral vector, and subsequently the SIN-lentiviral vector which has an advantage in targeting non-dividing cells, such as the primitive HSCs. The developed SIN-lentiviral vector has elicited a new wave of clinical trials for PIDs in recent years. The outcome from clinical trials for X-SCID, adenosine-deaminase deficiency (ADA-SCID), Wiskott-Aldrich-Syndrome (WAS) and X-linked chronic granulomatous disease (X-CGD) have demonstrated clinic efficacy with no evidence of vector-related toxicity observed clinically or by molecular analysis.[4–8] Overall, the results from those recent clinical trials showed a significant improvement in the safety profile and the efficacy compared to gamma-retroviral vector conducted gene therapy.

Recent fast development in genome editing using engineered nucleases has strategically transformed the idea of gene therapy for monogenic diseases including in hematopoietic stem cells.[9–11] The technique enables to edit genome at a specific locus leading to corrected gene expression under the control of endogenous regulatory machinery. This will obviate the problem of un-regulated transgene expression from viral vector in gene therapy. Therefore, the autologous HSCs transplantation combined with gene editing would provide an ideal therapeutic option for the treatment of congenital blood diseases and would become a major development in gene therapy filed in the future.

Gene therapy clinical trials for ADA-SCID, SCID-X1, X-CGD and WAS

Since the initiation of the first gene therapy trial over two decades ago.[12,13] More than 150 PID patients who did not have a matched donor have been treated by gene therapy (GT) worldwide. The majority of them have demonstrated clinical benefit. The significant developments have been made in the field of GT in last decade, including a safer vector platform, the automated CD34 + HSC purification and optimised conditioning regimes. Thus, the safety and efficiency of gene therapy has been largely increased.

Adenosine deaminase deficient (ADA)-SCID

ADA-SCID is an autosomal recessive disorder, and one of the most common severe forms of SCID. The deficiency in adenosine deaminase results in the accumulation of toxic metabolites in cells leading to impaired development of functional T, B and NK cells. The typical manifestations of ADA-SCID patients presented in the clinic are repeated and persistent infection from infancy which can be lethal without early clinic intervention.[14] In addition, the accumulation of toxic purine metabolites can also cause variable non-immunological abnormalities affecting the skeletal, lung, liver, gastrointestinal and nervous system, which can be difficult to handle clinically.

The treatment options for ADA-SCID include allogeneic hematopoietic stem-cell transplantation (HSCT), enzyme replacement therapy (ERT), or autologous gene therapy.[15] Although HSCT without conditioning is recommended as first-line curative treatment, the success rates are significantly reduced in patients who have an infection at the time of transplantation.[15–17] The administration of polyethylene-glycol-conjugated ADA (PEG-ADA) proved to be an effective tostabilize patients in short-term, therefore it has been used as a bridging therapy to promote immune recovery before a suitable treatment can be implemented. However, the long-term effects of PEG-ADA are often to be partial and not sustained over time due to reduced thymic output, increased spontaneous apoptosis and oligoclonal B cells. The cost of administration of PED-ADA is extremely high if maintained long-term.[18]

Because of the limitations of HSCT and ERT, gene therapy has been explored as a potentially curative treatment for ADA-SCID. In the initial clinical trials, the gamma-retroviral vector containing ADA cDNA driven by the viral promoter, long terminal repeat (LTR) was used.[12,19–21] The patients enrolled in those trials did not have the conditioning regimen, but continued to receive PEG-ADA in order to avoid the risk of a further deterioration of immune function. Although those early trials showed some efficacy with no observed genotoxicity, low vector marking cells were detected and failed to provide clinical benefit compared to that of ERT. The results indicated that continuously administrate ERT may impaired the selective growth advantage of gene-corrected cells. Therefore, some key changes have been made in the later trials, including the discontinuation of ERT after the GT, and the introduction of the non-myeloablative conditioning to promote engraftment into bone marrow. Eighteen patients were treated with this approach from 2000 to 2011 and an overall survival was 100% over 2.3—13.4 years (median, 6.9 years).[22] Gene-modified cells were stable within all multiple lineages throughout follow up. The immune reconstitution was demonstrated by normal level of T-cell subsets with sustained proliferative capacity, and the evidence of thymopoiesis. The most of treated patients were off immunoglobulin replacement therapy. Another clinical trial using the same approach was conducted in the UK, in which four out of six treated patients showed recovered immune function from gene-corrected cells. The adequate immunoglobulin production was shown in three

subjects.[23] Apart from those, a cohort of 16 patients was also treated in the United States.[24,25] Among them, the three of six patients treated by Candotti et al. remained well without supplement of ERT, showing gene marking 1%-10% in PBMC and the level of ADA enzyme activity in PBMC reached to near or to the normal range.[24] A similar efficacy was also shown in the trial led by Shaw et al. in which ten patients were enrolled.[25] In this trial, except one 15 year-old patient, nine patients were off ERT, showing normal level of ADA activity in PBMC, improved lymphocyte numbers, and normal proliferative responses to mitogens. Three patients had discontinued intravenous immunoglobulin replacement. Two patients who did not receive conditioning demonstrated delayed partial immune reconstruction and moderated etoxification of purine metabolites. This highlighted the importance of non-myeloablative conditioning for achieving clinical efficacy.[26] Based on the promising results obtained from those trials, the medicinal product Strimvelis received marketing regulatory approval in Europe for patients affected by ADA-SCID without a suitable HLA matched donor in 2016. This is the first ex vivo GT product to receive regulatory approval in the world.

Although there has been no insertional oncogenesis or leukemic proliferation observed in all >40 ADA patients treated with gamma-retroviral vector, the concern regarding potential leukemogenesis has led to developing SIN-lentiviral vector (SIN-LV) with a codon-optimized human ADA cDNA under the control of the short form elongation factor-1α promoter (LV-EFS-ADA).[27] Compare to gamma-retroviral vector, SIN-LV has some advantages, such as more favorable genome insertion profile, lack of intrinsic retroviral enhancer and higher efficiency of gene transfer.[28,29] The LV-EFS-ADA vector has been currently used in ongoing ADA-SCID clinic trials in the UK and USA.[7] Early indications in those more than 30 treated patients showed an excellent efficacy and no associated genotoxicity up to 3 years follow-up (H.B.Gaspar and D.B.Kohn, personal communication). Of note, ERT is continued through the first month after GT in the current trials. This is based on the findings in the murine model, in which there was no difference in gene-marked in lymphocytes between ERT and non-ERT group.[30] Using ERT in early stage after GT may help in maintaining a detoxified environment which may improve engraftment. So far, ADA-SCID patients treated with GT worldwide from different centres showed no severe adverse events related to insertional mutagenesis. This is in sharp contrast with those from GT trials for other PIDs such as X-SCID, WAS and X-CGD, where a very similar vectors were used. This suggests that disease background maybe a critical factor in influencing leukemogenic potential in gamma-retroviral GT. Comprehensive analysis of retroviral integration sites (RISs) in 15 ADA-SCID patients treated with LTR-intact gamma-retroviral vectors demonstrated the insertion site profiles near the proto-oncogenes (including *MECOM* and *LMO2*).[31] The subtle mutagenic clonal disturbances were also detected. But these clones remained stable over several years and never dominantly expanded. The reasons for the disease-specific difference in toxicity are not clear. It has been postulated that ADA enzyme production may cross-rescue ADA-deficient lymphocytes, thereby placing lower replicative stress on gene-corrected cells.[32]

X-linked SCID (SCID-X1)

SCID-Xl is the most common form of SCID and is caused by mutations in the *IL2RG* gene leading to defective expression of the common gamma chain (γc), a subunit shared by cytokine receptors including interleukin (IL)-2, 4, 7, 9, 15 and 21 receptor complexes.[33] SCID-Xl is characterized by low number or absence of circulating T and NK cells and the loss of function of B cells.[34] The observation that lymphocyte progenitors possess selective advantage over the deficient progenitors makes SCID-Xl a strong candidate for gene therapy.[35]

The first clinical trial of gene therapy for SCID-X1 was performed in Paris between 1999 and 2002. Ten children under 1 year old were enrolled. The trial was conducted using a amphotropic murine leukemia virus (MLV)-based gamma-retroviral vector in which γc gene expression was driven by the viral promoter LTR.[36] Another clinical trial was carried out in London with ten SCID-X1 patients enrolled, in which a gibbon ape leukemia virus (GALV) —pseudotyped gamma-retroviral vector was used.[37] Both trials omitted preconditioning before gene therapy showed the successful recovery of the functional T cell compartment in 17 patients. The polyclonal and functional T cell receptor repertoires were detected in the long term follow-up period. The restoration of humoral immunity was found to be partial but sufficient to withdraw immunoglobulin replacement therapy in some patients. Long-term NK cell marking was very low, which might due to weaker proliferation or survival capacity of the NK progenitors compared with that of T cell progenitors. Unfortunately, five out of the 20 SCID-Xl patients treated in France and UK developed acute T cell lymphoblastic leukemia within 2—5.5 years after GT.[38,39] In four of five patients, the gamma-retroviral vector in the leukemic clone was integrated near the LIM domain-only 2 (LMO2) proto-oncogene and up-regulated its transcription.[39] Four patients entered remission following standard chemotherapy while one patient died due to refractory leukemia. As part of the clinical trial in Paris and London, SCID-X1 GT was attempted in 5 older patients (age 10—20 years) who failed to show any significant clinical benefit.[40,41] This may due to an age-related loss of thymopoietic capacity or the impact of long period chronic infection and GVHD.

To improve the safety profile of GT for SCID-X1, a self-inactivating gamma-retroviral vector was developed with deletion of the LTR enhancer and incorporated a human elongation factor-1α in driving the transgene expression.[42,43] The SIN gamma-retroviral vector has been used in nine boys in parallel European and American trials without preparative conditioning.[44] Eight of the nine children were alive after 12.1—38.7 months of follow-up, while one died from an overwhelming adenoviral infection prior to immune reconstitution. Of the remaining eight patients, seven had recovery of the functional T cells which led to alleviating infections, but humoral function was not restored in these patients. More importantly, integration analysis revealed significantly less clustering near the lymphoid proto-

oncogenes compared with those treated by LTR-intact gamma-retroviruses. This indicates that the modified SIN gamma-retroviral vector has improved safety profile.

More recently, SIN-LV using an EF1α promoter driving a codon-optimized γc cDNA flanked by 400-bp chicken insulators was applied to a trial in five older patients (aged from 7 years to 23 years old)who have failed haplo-identical HSCT.[5] The follow-up data from two older patients (23 and 22 years old respectively) who had non-myeloablative busulfan conditioning showed the selective expansion of gene-marked T, NK, and B cells, which is associated with the sustained restoration of humoral responses to immunization within 21 months of therapy. Similar gene marking levels was also shown in three younger patients (7, 15 and 22 years old respectively) with the same conditioning, albeit with only 6—9 months of follow-up. Lentiviral gene therapy with reduced-intensity conditioning appears safe and could improve the restoration of humoral immune function even to older SCID-X1 patient. So far, no vector-related adverse events have been reported from those treated patients. Although the follow-up duration is limited, these results are very promising for SCID-X1 gene therapy treatment.

Recently, Humbert O et al tried to explore *in vivo* gene therapy platform by directly infusion of foamy virus (FV) vector containing γc cDNA into a SCID-X1 canine model following the mobilization of CD34 cells by G-CSF and AMD3100 without conditioning.[45] Unlike the LVs, FVs are resistant to human serum inactivation,[46] therefore it is suitable to be used *in vivo* delivery. FVs are also potentially safer than the LVs due to their favorable integration profile.[47] In this study, improved thymic output and broad TCR repertoire were detected in mobilized FV-treated animals. In addition, STAT3 phosphorylation in CD3+ lymphocytes was also detected from mobilized FV-treated animals compared with non-mobilized SCID-X1 animals. Although the study has demonstrated a novel alternative *in vivo* treatment of SCID-X1, the efficiency achieved in this study may not be sufficient for clinical benefit and need to be improved for the application.

X-CGD

Chronic granulomatous disease (CGD) is a rare inherited autosomal recessive or X-linked disorder predominantly affecting neutrophil function. The condition arises from mutations affecting any one of the five genes encoding phagocytic oxidase proteins which form the subunits of the NADPH oxidase enzyme complex. This oxidase enzyme complex is directly responsible for mitochondria-independent consumption of oxygen during phagocytosis associated with the production of reactive oxygen species (ROS), and the activation/releasing of antimicrobial proteases to eliminate invaded microbes.[48] CGD patients fail to generate this "respiratory burst" and are consequently susceptible to recurrent life-threatening of bacterial and fungal infections. The mutation in the X-linked *CYBB* gene encoding the gp91phox component of the NADPH oxidase complex constitutes two thirds of CGD (X-CGD) with the greatest threat of infection and mortality.[49,50] The available therapies for CGD include a life-long antibiotic prophylaxis, the recombinant interferon (IFN)-γ treatment and myeloablative allogeneic HSCT. Although myeloablative allogeneic HSCT using a closely matched related or unrelated donor offers the treatment option for children with CGD, the side effects such as graft-versus-host disease and inflammatory exacerbations are often seen indicating the need for alternative therapies.[51]

The rationale for gene therapy of CGD patients lacking a HLA-matched donor is well-established since a small amount of normal superoxide production could result in significant clinical improvement. However, gene therapy for CGD has encountered particular challenges. One of the challenges is that expression of the wild-type gene in corrected cells do not confer a selective survival advantage. Furthermore, circulating neutrophils have a short life-span, suggesting that a large number of corrected HSCs are required for achieving clinical efficacy. Apart from those, chronic inflammatory may have a detrimental effect on HSC numbers and viability after ex vivo manipulation.[52,53] The first GT clinical trials for CGD were performed in five patients in USA,[54] using a gamma-retroviral vector without any preconditioning. Although there were no severe adverse events, gene marked cells were detectable at low levels for a few months after treatment and failed to confer any long-term clinical improvement. Subsequent clinical trials have been conducted in several centers worldwide using reduced-intensity conditioning.[52,55—57] Twelve patients were enrolled and the significant clinical benefits were seen in the early phases of the treatment, with the majority of patients showed the clearance of pre-existing infections. However, the clinical benefit was only transient, and gene marking rapidly decreased after 3 months. A trial performed in Germany using a spleen focus-forming virus (SFFV)-derived LTR-based gamma-retroviral vector with non-myeloablative conditioning showed that two patients developed unexpected high level of functional neutrophils. This oligo clonal expansion of neutrophils was caused by the vector insertion into MDS/EVI1 proto-oncogene and subsequent trans-activation by the SFFV LTR. Unfortunately, both patients developed myelodysplasia with gradual loss of oxidase function in the gene marked cells caused by methylation of the retroviral promoter.[55] One patient died 2.5 years post-therapy of severe sepsis associated with myelodysplasia and the second patient died following unrelated donor bone marrow transplantation.[58] Of the two children treated in Switzerland, one developed myelodysplasia while the other showed the clonal expansion without myelodysplasia. Both patients were rescued with allogeneic stem cell transplantation.[59] One of lessons learned from these early X-CGD GT trials is that the conditioning is of particularly important for X- CGD gene therapy. And the leukemic events occurred in three treated patients emphasized the importance of developing a safe vector.

To reduce the risk of the mutagenesis and increase long-term efficiency, a SIN-lentiviral gene therapy approach for X-CGD has been developed, in which gp91phox driven by a myeloid specific chimeric promoter was used. The chimeric promoter was created by the fusion between c-fes and cathepsin-G-proximal regulatory sequences and showed preferentially active during terminal myeloid differentiation. Consequently, it confers higher levels of gene expression in differentiated myeloid cells rather than in

non-myeloid lineages or in human hematopoietic progenitors, where mutagenic influences are least desirable.[60] There are currently two parallel GT trials for X-CGD using this vector with myeloablative busulfan preconditioning. So far, the results from 7 severe X-CGD patients were collected, showing stable persistence of 10—50% oxidase positive in neutrophils. No molecular evidence for clonal dysregulation or gene silencing through CpG dinucleotide methylation was found (Kohn and Thrasher personal communication).

WAS

Wiskott—Aldrich syndrome is a rare and severe X-linked immunodeficiency caused by mutations in the WAS gene which encodes a 502-amino-acid protein called the Wiskott-Aldrich protein (WASp).[61,62] WASp regulates the actin cytoskeleton in most hematopoietic lineages and is consequently important for normal function of many immunological processes. Clinical manifestations of WAS include microthrombocytopenia, recurrent infections, and eczema. Patients also display an increased incidence of autoimmunity and a risk of developing lymphomas.[63] Despite improvements in clinical care, the outcome for the patients with classic WAS remained poor with a median life expectancy of 15 years for those who do not receive definitive treatment. For many years, allogeneic HSCT is recognized as the only potential curative therapy for WAS and the success rate is determined by availability of HLA-matched donors and the clinical score at the time of treatment.[64] Autoimmune complications are more frequent in those who do not achieve complete chimerism post allogeneic transplant.[64] Given these factors, gene therapy has become an alternative treatment for WAS patients without suitable donors or at high risks of complications.

The first GT clinical trial for ten WAS patients was performed in Germany, using a gamma-retroviral vector following reduced intensity busulphan conditioning.[65,66] Nine patients exhibited restored WAS expression in multiple lineages, showing the increased proportion of corrected lymphocytes, improved platelet count and the resolution of bleeding, eczema and auto-immunity. Unfortunately, seven patients develop acute leukemia due to insertional transactivation of proto-oncogenes LMO2, MDS1 or MN1 by a strong enhancer element within the viral LTR, between 16 months and 5 years post-therapy.[66] Affected patients were treated with chemotherapy and allogeneic HSCT, but two patients died from leukemia. Therefore, GT using gamma-retroviral vector for treatment of WAS carries a high level of insertional oncogenesis risk.

More recently, a SIN lentiviral vector consisting of the endogenous 1.6-kb human WAS promoter has been chosen for clinical trials using a reduced-intensity preconditioning regimen in Italy, France, UK and USA.[4,6,8] A clinical trial in Italy enrolled three WAS patients. All of three treated patients showed a stable engraftment of genetically corrected cells as well as improved thrombocytopenia, eczema, and reduced infectious episodes. Vector integration analysis revealed highly polyclonal and multi-lineage hematopoiesis resulting from the gene-corrected HSPCs. No aberrant clonal expansion was observed after 20—32 months therapy. Subsequently, seven patients with severe Wiskott-Aldrich syndrome were enrolled in France and UK with follow-up ranged from 9 to 42 months.[4] Six patients showed the improved immune function and clinical manifestations. One patient died from preexisting drug-resistant herpes virus infection at seven months after the treatment. The clinical manifestations of eczema and susceptible to infections were resolved in all 6 patients. Autoimmunity improved in 5 of 5 patients. No severe bleeding episodes were recorded after treatment, and all 6 surviving patients were free of blood product support. The vector-related toxicity was not observed clinically or by molecular analysis. Of note, neither trial above resulted in reconstitution of normal platelet numbers. However, among four patients treated in USA trial, (Julia I Chu et al., 2015), one patient who received the highest cell dose, with high VCN has gained a normal level of platelet count and high level gene marking in myeloid cells. This highlights that the high cell dose with high VCN can lead to an increased efficacy. More recently, a 30-year-old WAS patient was treated in UK.[6] The patient was able to discontinue immunosuppression and exogenous immunoglobulin support, with improvement in vasculitis disease and proinflammatory markers after GT. This indicates that GT is a viable strategy for adult WAS patients with severe chronic disease complications where allogeneic procedure could present an unacceptable risk. So far, more than 20 patients have been treated using SIN lentiviral vector in those four centers and the results are promising, though platelet recovery has been variable in those trials.[67] Long-term follow up is warranted to confirm the safety and efficacy of lentiviral GT as an alternative treatment option for this disease.

Further improvements

The outcome clinical trials of gene therapy (GT) carried out so far is promising. However, it may appear premature to claim that GT is ready to enter the mainstream of medicine. More work need to be done to evolve this novelty therapy to becoming a standard treatment for all patients. So far, only few medical centres are able to perform the treatment, which is almost not accessible for PID patients outside of those countries. The funding for those centres are mostly relies on charities and research societies which make the coordination of resources are extremely hard to bring down the cost of the treatment. The high cost and limited access to the treatment centres are the major challenging for many devastating PID patients worldwide. Recently, the capitalization of GT involved with the biotechnology firms may facilitate the development of GT treatment with their financial support and the strength in research and development. However, it is still a long way to establish a commercial centre which could supply a ready GMP cryo-preserved cell product that can be accessed by high number of clinical centres on a wider geographical area.

The outcome of clinical trials have demonstrated that pre-conditioning is an essential for achieving a better engraftments of gene corrected cells. Although the low intensity conditioning used in GT has been well tolerated by the patients, the drugs used in the conditioning may

imposed a long-term risk of cytotoxicity to the patients, particular to those with pre-existing organ damage. Thus, less toxic alternative HSC depletion strategies need to be established. Palchaudhuri et al.[68] recently developed an alternative safe conditioning approach based on the antibody strategy which selectively targets HSC-surface markers CD45 (CD45-SAP). The authors demonstrated that the CD45-SAP enabled efficient (>90%) engraftment of donor cells and the fully correction of a sickle-cell anaemia in murine model of the disease, with a minimal toxicity to non-hematopoietic tissues. Thus this non-genotoxic conditioning method may provide an attractive approach to current conditioning regimens.

The efficiency of the lentiviral vector can be affected *in vivo* by the position effect mediated transgene silencing if the vector integrated into a repressive-chromatin in cell genome. Therefore, the refinement of the vector that resist to the transgene silencing will confer a stable and efficient transgene expression. This problem can be improved by incorporating the regulatory elements such as the insulators[5,69,70] or chromatin-opening element[71,72] into the vector. However, the further validation is needed for their application in GT. For some PIDs that lack of selective advantage of gene corrected cells, the efficient transduction is of important for clinic efficacy. Some compounds (Retronectin, Protamine sulfate, plus two recent reported cyclosporine H and Lentiboost) have been shown enhancing gene transduction with a high efficiency.[73,74] However, a cross comparison of those compounds is necessary in order to define the most effective and safety condition that can be applied to the GT clinical trial.

Unregulated transgene expression in the current gene therapy could be a problem for certain PID patients where precise gene regulation is required for the efficacy. The genome editing technology developed recently enables to edit genome at specific locus leading to corrected gene expression under the control of endogenous regulatory machinery. Therefore, this technology has opened a new platform for the potential treatment of hematopoietic disorders.

Gene editing in hematopoietic progenitor stem cells (HSPCs)for potential therapy

The genome editing using engineered nuclease has strategically transformed the idea of gene therapy for monogenic diseases including in HSPCs.[9–11] The genome editing technology enables to create a site specific double-strand break (DSB) by the engineered nucleases that programmable triggering the cell's endogenous repair machinery to edit the genome in a site-specific manner via the non-homology end joining repair (NHEJ) and the homology directed repair (HDR) mechanisms.[75] The approach allows the precise alteration of the disease-causing allelesat the specific locus making it a permanent event that maintains the phenotypical gene expression under the control of endogenous regulatory elements.

Over the past decade, three major classes of engineered nucleases have been used for genome editing, including zinc-finger nucleases (ZFNs),[76,77] transcription activator-like effector nucleases (TALENs)[78,79] and CRISPR–Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9).[80–83] ZFNs and TALENs are fusions between arrays of ZF or TALE DNA-binding domains and the dimerization-dependent FokI nuclease domain. Both of ZFN and TALEN nucleases exclusively rely on protein–DNA interactions to mediate site-specific recognition of genomic DNA sequences which requires complex protein engineering for each new target. By contrast, CRISPR–Cas9 nuclease is a RNA-guided endonuclease. Through the guidance of a 23 nucleotides RNA linked to CRISPR-domain (gRNA), CRISPR-Cas9 finds the complementary protospacer DNA target in a genome where it cuts the double stranded DNA precisely 3 base pairs upstream of a PAM (Protospacer Adjacent Motif). The broken DNA ends generated by those nucleases are repaired either by NHEJ resulting in small insertion/deletions (indels) to disrupt target allele, or by HDR to precisely replace desired nucleotides with delivery homologous DNA template. Compared to ZFNs and TALEN, the CRISPR/Cas9 system has rapidly become the most promising genome editing tool with demonstrated advantages including simplicity, easy programming, low cost and potential multiplexed editing.[82,84–87] Although the genome editing holds tremendous promise for the developing novel gene therapy, the technique has been shown to be more refractory in HSPCs than any other cell types due to their quiescent status and prone to DSB induced toxicity. However since the first publication using ZFNs-mediated editing in HSPCs,[88] substantial developments have been made in last few years to circumvent the problems.

Optimization of gene editing efficiency in HSPCs

Ex vivo expansion of HSPCs

Since all nucleases targeted gene editing occurs through cell cycle progress, increasing stimulation of HSPCs ex vivo can make them more permissive to editing components. However, their manipulation can also promote cell differentiation. To circumvent this, the compounds that agonist HSPC self-renew while maintaining their primitive phenotypes have been discovered and applied to the cell culture.[89–91] Using the compounds in HSPCs culture, researchers have achieved significantly increased rate of edited HSPCs *in vitro* and increased engraftment *in vivo*.[88,92] In a recent study, Psatha et al. have described 5 days HSPC culture condition, in which StemRegenin 1(SR1) was used in combination with a small molecule Ly2228820 (SL), a p38-MAPK14 inhibitor.[93] Using this culture condition, they have successfully expanded highly engraftable $CD34^+/CD38^-/CD90^+$ primitive HSPC cells. They then tested if this protocol could also expand edited HSPCs effectively. To this aim, they cultured edited HSPCs for additional 5 days after editing, and found that edited $CD34^+/CD38^-/CD90^+$ primitive HSPCs can be effectively expanded *in vitro* without any loss of editing efficiency. Moreover, the expanded edited cells led to more than 2-fold higher engraftment compared to their unexpanded counterparts.[94] The study highlights a possible way to obtain sufficient engraftable HSPCs by expanding cells before, or after editing ex vivo in presence of SR1 and SL. However, the convincing evidence on long-term *in vivo* engraftment from significantly expanded HSPCs is needed to ensure the absence of any oncogenic burden associated with *ex vitro* expansion.

Delivering the editing components

In clinical application setting, the approach for delivering nucleases or other components into HSPCs should be transient to avoid the cytotoxicity and aberrant immune responses engendered by prolonged endonucleases activity. Therefore, a "hit-and-run" approach is used. The mostly used method for delivering DNA or RNA encoding engineered nucleases is via nuclear transfection. One of main concerns in transfection of plasmid DNA to HSPCs is its potential random integration of the plasmids into the genome which could lead to genotoxicity in HSPCs and their progenies. And also DNA related cytotoxicity, such as cyclic GMP-AMP synthase induced pathway[95] could also lead to high toxicity in primitive HSPCs. Therefore, transfection of mRNA encoding nucleases synthesized *in vitro* has become an optimal alternative approach.[96,97] From recent studies it has emerged that the mRNA transfection approach has indeed provided an increase in the efficiency of genome editing in HSPCs.[98–100] In addition, Cas9 can be delivered as a protein or as precomplexed ribonucleoproteins (RNPs) by mixing gRNAs with the Cas9 protein.[97,101] This approach helps to protect the gRNAs from degradation, and to reduce cytotoxicity caused by naked RNA-stimulated innate immunity. The improved editing efficiency based on such approach has been achieved in targeting HSPCs as shown in recent studies.[99,100,102,103]

Apart from above components, a safe and efficient delivering DNA donor template into edited cells is crucial for achieving HDR process. Several donor template platforms have been used. A single-stranded DNA oligonucleotide (ssODN) donor has been shown as a simple and effective approach in genome editing for correction of single-nucleotide mutation in HSPCs.[104,105] Integration defective lentiviral vector (IDLV), that allow incorporation of a large DNA template, has been used for ZFN-mediated genome editing to target mutation in the *IL2RG* mutations and ADA genes.[88,106] However, those early studies showed a limited gene targeting efficiency in HSPCs, suggesting that IDLV could be even more cytotoxic to HSPCs. The efficiency of IDLV as a donor template in HSPCs can be significantly improved by using cyclosporine H, which is showed in a recent study.[73] Recombinant adeno-associated viral vectors (rAAVs) have been shown to naturally mediate HR in mammalian cells without stimulating DSB.[107–109] Hence, rAAV vectors are emerged as ideal delivery approach due to their wide range of tropism, high transduction rate and very low immune response. In particular, the rAAV6 vector has been shown to provide more efficient and robust genome-editing in HSPCs than other delivery vectors shown in recent therapeutic potential studies.[98–100,105,107] To improve the packaging capacity of AAV6, Bak et al.[110] have developed a dual-AAV6 donor vector system that enables delivering a large transgene cassette up to 6.5 kb into primary T cells and HSPCs with long-term repopulation capacity. Overall, the conditions for delivery the components used in gene editing should always be optimised for each targeted gene to achieve most efficient targeting and minimum cytotoxicity. A comprehensive detailed protocol using CRISPR/Cas9 with rAAV6 as templet vehicle for HDR-targeted editing in HSPCs has been published by Bak and Daniel recently,[111] which could be also served as a guide for implement gene editing technique for other nucleases.

Improve HDR

Unlike NHEJ repair pathway which occurs throughout the cell cycle, HDR is restricted to the S/G2 phases of cell cycle which makes this process much less efficient than NHEJ.[112,113] Therefore, inhibiting nuclease activity during the G1/M phases and synchronizing cells at the S/G2 phases may improve HDR efficiency. The concept has been experimentally tested by Gutschner et al.[113] In their study, the hGem-Cas9 system was generated by incorporating the human geminin domain which allows the nuclease to be ubiquitinated and degraded by APC/Cdh1 complex in G1 and late M phase, therefore leading to increased hGemCas9 activity in S/G2 phases. Using this cell-cycle-tailored hGemCas9 system, Gutschner et al have achieved an increased rate of HDR up to 1.87 fold compared to wild-type Cas9 in cell lines. A further development based on this approach in HSPCs was published recently by Lomova et al.[114] In their study the hGemCas9 was used in combination with a cell synchronization compound RO-3306 which functioning in transiently arresting cells at S/G2 phase via inhibiting CDK1.[115] It was shown from Lomova's study that the ratio of HDR/NHEJ was increased to four-fold in human CD34 + cells compared to the controls *in vitro*, with a significant improvement engraftment observed *in vivo*. The improved HDR can also be achieved by directly inhibiting the NHEJ pathway through targeting of the DNA ligase IV, a key enzyme in the NHEJ pathway, using the inhibitor Scr7.[116,117] Although increased HDR efficiency has been achieved in human cell lines and cancer cells, so far, it has not been assessed in human HSPCs.

The assessment off-target sites

Although ideal engineered nucleases would have singular genome-wide specificity, unintended off-targets can occur, particularly at loci with homology to the intended target site. Several the off-target detection methods have been used in HSPCs gene editing studies. An early developed assay is based on using the *silico* prediction off-targets sites sharing degree of similarity to the on-targets sequences.[118,119] This method is still the most used in the HSPCs editing studies due to its simplicity. However the fundamental limitation with this approach is that it is not designed to identify off-target sites in an unbiased manner as the sites that do not fit the computational criteria will not be identified.

To achieve unbiased off-target detection, cell based genome-wide assays have been developed. Among them Integrase-defective lentiviral vector (IDLV) capture assay was designed to capture IDLV linked with GFP into sites of nuclease-induced DSBs. Then clustered sites of integrations are recovered by linear amplification-mediated PCR and mapped using high-throughput sequencing.[120] Although this method can directly identify DSBs in living cells, it is relatively insensitive due to the low absolute integration efficiency of IDLV, which could be overcome by positive selection of transduced cells.[120] On the other side, the assay may have high background due to random integration of IDLVs into cellular genomes even in the absence of nuclease-induced DSBs.[120]

Whole genome sequencing (WGS) has been proposed as an unbiased method for defining engineered nuclease specificity. Although this method is useful for the analysis of single-cell clones,[121] it lacks sensitivity, particularly for those low frequencies off-target in a cell population.[122]

With existing deep sequencing technology, it is impractical to perform WGS on millions of cellular genomes, and it is inadequate to confirm the off-target sites at $< 0.1\%$ in a cell population.[82] Considering the limitations of those off-target assays, it would be necessary to use combined approaches as shown in Kuo' study[100] to ensure confidence in safety of therapeutic gene editing in HSPCs.

Therapeutic potential of HSPC gene editing

Non-homologous end joining-based strategy

NHEJ DNA repair pathway is an error prone repair process that leads to the alteration of nucleotide sequencing at the specific site via in-frame deletions or insertion (indels). Sine it is the preferentially used pathway to repair DSBs, it has become a viable genome editing option for correcting gene mutations. Two loci, CCR5 and BCL11A, have received the early attention as their potential therapeutic benefits via NHEJ process. The concept of editing CCR5 was intrigued by the report that the transplantation of a donor HSCs with a naturally occurring *CCR5* mutation confersa loss of detectable HIV-1 RNA and proviral DNA in a HIV patient.[123] Holt et al. first demonstrated the successful disruption of CCR5 gene using ZFNs.[124] In their study, NSG mice transplanted with ZFN-modified HSPCs underwent rapid selection for CCR5(−/−) cells and showed significantly lower HIV-1 level compared to the controls when challenged with CCR5-tropic HIV-1. Several studies published later have also demonstrated the feasibility of this approach engendering resistance to HIV infection in *in vivo* model.[125–127] Among them, DiGiusto et al. conducted a pre-clinical study to assess efficacy and safety of the ZFN-based CCR5 disruption by delivering CCR5-specific ZFN-mRNA to normal adult HSPCs. The authors demonstrated effective biallelic CCR5 disruption 40–60% of cells, and in up to 72.9% of modified colony forming units from edited HSPCs. The edited HSPCs preserved long-term multiple lineage potential *in vivo* with no demonstrated potential for tumorigenesis or leukemagenesis. Based on this, further safety and feasibility studies are ongoing in subjects infected with HIV-1 (NCT02500849@clinicaltrials.gov).

Targeting of the BCL11A genomic locus in HSPCs via NHEJ has been developed as a potential treatment for β-hemoglobinopathies, either β-Thalassemia or sickle-cell disease (SCD), which are inherited monogenic blood disorders caused by the mutations in the β-globin gene.[128] The reduction in the severity of both conditions achieved through induction of Fetal haemoglobin (HbF)[129] led to the discover BCL11A as a repressor of HbF expression,[130] which could be targeted to induce HbF in HSPCs. To this aim, Bjurstom et al. employed the genome editing strategy aimed to disrupt the *BCL11A* exon2 in HSPCs using the ZFNs, TALENs or CRISPR-Cas9.[131] In this study it was shown that the ZFNs gave rise to more allelic disruption in the targeted locus which is associated with increased levels of HbF in erythroid cells derived from nuclease-treated $CD34^+$ cells *in vitro*. However, a low level of disruption in the *BCL11A* gene (4%) was observed in bone marrow of animals after transplantation of edited cells into NSG mice. Using a ZFN-editing approach Chang et al.[132] compared targeted disruption of BCL11A expression, either within exon 2 or at the GATAA motif contained in the erythroid-specific BCL11A enhancer region. Their study showed that the allelic disruption of GATAA not only gives rise to robust long-term engraftment and consequent lyelevated level of HbF expression in erythroid cells, but also prevents aberrant erythroid enucleation seen in the *BCL11A* exon2 ablation. Using the same strategy, a comprehensive preclinical study has been carried out in HSPCs from adult donors and two patients with β-Thalassemia Major.[94] The modification of the GATAA motif in mobilized CD34 + cells from β-thalassemia patients resulted in a readily detectable increased HbF, leading to an improved phenotype that likely give a survival advantage to maturing erythroid cells. The phase1/2 clinical trial for correcting the β-thalassemia phenotype is currently being evaluated by the same group.

Homologous recombination based strategy

In the great majority of genetic blood diseases, an HDR-based editing strategy is required to correct the genotype. The process is much more challenging than NHEJ-based pathway due to its low efficiency, particular in targeting primitive HSPCs. However, a promising progress in targeted integration in HSPCs for some PIDs has been made in recent years.

SCID-X1. The first attempt using ZFNs for gene knock in *IL2RG* in HSPCs was conducted by Genovese et al.[88] In this study, two genomic loci, *AAVS1* "safe harbour" or *IL2RG* locus were targeted with a GFP cassette delivered via IDLV vector. Although they achieved 24–26% of editing, only 5% GFP+ colonies were found in colony-forming unit assay. At 8 weeks after transplantation of edited CD34 + cells into NSG mice, only 2% GFP+ cells were found in primitive and committed progenitors in the bone marrow of animals. To improve gene target efficiency, Genovese et al. optimised the culture condition by extending HSPCs activation time to make them more permissive to the editing and by adding compounds into the culture medium to preserve HSPC's stemness.[88] The modified protocol then was used in HSPCs with delivering IDLV vector containing the exons 5–8 *IL2RG* cDNA and a PGK-GFP cassette and showed increased GFP+ cells (≥2-fold) in primitive cell population in NSG mice received edited cells at 14 weeks. Using improved protocol Genovese et al. performed the *IL2RG* gene correction in HSPCs derived from a SCID-X1 patient. The authors found a 3% GFP+ cells in primitive HSCs and up to 11% GFP+ in committed progenitors *in vitro*. The CFU assay yield 3 GFP+ colonies out of 100 scored, with a myeloid progeny colony showing reconstituted normal IL2RG protein expression. The data from this study highlighted the problem associated to primitive HSCs targeting for homologous recombination.

A recent advancement in *IL2RG* targeting has been demonstrated by the same group.[99] In order to establish therapeutic potential of gene editing on *IL2RG*, a preclinical model, humanise SCID-X1 mouse was used to stringently evaluate efficacy and safety in a pre-clinical setting. After obtained convincing functional evidence in gene editing in this model, Schiroli and colleagues optimised human HSPCs

editing strategy via targeting the intron 1 *IL2RG* site which allows correct the majority mutations in *IL2RG*. By further modifying a pair of six-finger ZFNs, they achieved high on-target activity and improved tolerability at increased doses. They have also modified the ZFN mRNA by incorporating the base analogs to prevent recognition via cellular sensors, which are associated with the activation of the interferon-responsive genes by exogenous RNAs. This modification resulted in a significantly reduced cytotoxicity caused by *in vitro* electroporation of the ZFN mRNA, leading to high HDR (25%) in HSPCs derived from a SCID-X1 patient. By switching to an AAV6 as a donor DNA delivery tool, they achieved up to five fold higher HDR-mediated gene editing in the most primitive CD34 + CD133 + CD90 + cells compared to the IDLV-based approach. It was also demonstrated that this optimised protocol could be transferable to the clinic scale, showing reproducible editing efficiency even in a large scale setting. More importantly, the edited cells preserved long-term engraftments in NSG mice, showing an average 12% HDR-corrected HSPCs at 16 weeks post-transplantation, which exceeded the threshold (10%) for fully reconstitute immune function at a standard transplant dose established in the their study.[99] The off-target assay did not detect significant amount of specific modifications above the threshold of sensitivity in any of the off-target sites identified previously by genome-wide screening for this set of ZNFs.[120] Based on these data, it would be interesting to see if the optimised protocol could lead to improved editing efficiency *in vivo* in HSPCs derived from the SCID-X1 patient, which could pave the way to the translation of HSPC gene editing into the clinic.

X-linked chronic granulomatous disease (X-CGD). Two recent studies published by De Ravin et al. presented promising results on targeted correction of the *CYBB* gene, encoding gp91^{phox}, for the treatment of X-CGD.[98,105] Their initial study[98] used ZFNs to target the integration of a *CYBB* transgene into a genomic "safe harbour"AASV1. This strategy could be a viable alternative to previous gene therapy attempts, where three gene therapy treated X-CGD patients developed myelodysplasia due to the integration of the retroviral vector at the MDS-EVS1 locus.[58] De Ravin et al. carried out extensive experiments to explore clinically-relevant protocols to deliver ZNFs and AAV6, with the aim to knock-in a promoter-less Venus reporter cassette into the AAVS1 locus. The results from their study showed an average 30% Venus+ HSPCs *in vitro*, and 10.8 ± 4.2% Venus+ cells among 40.1 ± 14.6% of human engraftment in bone marrow of NSG mice (n = 16) at 17 weeks post-transplantation. Using their optimised approach, they then targeted HSPCs derived from X-CGD/gp91phox patients with donor template constructs containing either a promoter less gp91phox (2A-2A-gp91), or gp91phox driven by a synthetic MND promoter (MND-91). Although both approaches showed a similar targeted integration efficiency (with ~15% gp91phox expression), a robust functional correction through the MND promoter, rather than the endogenous *PPP1R12C* promoter was obtained showing significant high MFI in gp91 expression and DHR oxidase activity in edited HSPCs *in vitro*. At 8 weeks post-transplantation of edited HSPCs into NSG mice, the analysis of gp91 expression cells in the human CD45 + cells in bone marrow showed 7 ± 4.2% and 10.7 ± 4.2% for the MND-91 and 2A-2A-gp91 respectively, which was about 10%–30% level of healthy uncorrected HSPC engraftment (35.6 ± 2.8% gp91 expressing cells). Since corrected X-CGD cells do not entail a selective advantage *in vivo*, the question is if the level of reconstituted gp91 expressing cells achieved in this study would be sufficient for the disease phenotype correction. The off −target was assessed in sorted human CD45 + cells from mouse bone BM and confirmed the potential off-target sites at a level below the detection assay used. The data presented in the study provided the first promising alternative approach for treatment of X-CGD. However, long-term efficiency in correcting gp91*in vivo* still remain to be established, and the safety issue regarding of disrupting the *PPP1R12C*in the AAVS1 site in stem cells also need to be carefully assessed.

In a later study led by the same group,[105] De Ravin et al. have achieved the targeted correction of an X-CGD-causing point mutation (C676T) using CRISPR/Cas9 in conjunction with a single strand oligo nucleotide (ssODN) as a donor template. The C676T mutation accounts for 6% of X-CGD patients and occurs at exon 7 of the *CYBB* gene resulting in a premature stop codon and an inactive gp91^{phox} protein. Following experiments to optimise the targeting of the mutational hotspot in the *CYBB* locus in normal CD34 + cells, they achieved 12–31% targeting rate indicated by gp91^{phox}in HSPCs derived from an X-CGD C676T mutation patient. They then assessed the functional gp91^{phox} in myeloid cells differentiated from edited HSPCs. Upon FMA stimulation, 19.3% of myeloid cells from the patient were positive for NOX2 activity compared to 80% from health control, and superoxide radical production from patient myeloid cells was one-third of the amount produced by normal health control. Analysis of the engraftment of gene-repaired X-CGD HSPCs transplanted into NSG mice showed stable engraftment of corrected cell and the production of functional mature myeloid cells differentiated from edited patient HSPCs for up to 20 weeks. The off-target analysis on computationally predicted off-target sites in edited X-CGD HSPCs revealed one single indel (>3 bp) at the RP11-454H19.2 gene. However, one single indel was also observed in the uncorrected healthy control CD34 + HSPCs, indicating that this could be due to amplification/sequencing errors at high level of coverage. Whole-exome sequencing at 800× coverage of corrected patient HSPCs also failed to detect any off-targets. Using the same approach, De Ravin et al. also tried to correct a second X-CGD patient harbouring the CYBB 676 mutation.[105] Although they achieved similar level of gene repair as in patient 1 *in vitro*, a less than 50% of corrected X-CGD HSPCs were observed after transplantation into NSG mice. This has highlighted the necessity of careful validation of all conditions at every level of editing procedures to ensure consistent outcome. Nevertheless, this study presented a viable approach to correct of a missense mutation in HSPCs

restoring physiological gene expression by endogenous regulatory regions.

X-linked hyper-IgM syndrome (XHIM). XHIM is a primary immunodeficiency caused by mutations in the CD40 ligand gene (CD40L) expressed on activated T cells. The mutated CD40L fail to bind CD40 on B cells thus affecting immunoglobulin class switch recombination resulting in the absence of IgG, IgA, IgE with a normal or elevated IgM. XHIM patients are susceptible to bacterial infection, and with development of autoimmunity and malignancies in some individuals.[133,134] XHIM can be treated by allogenic HSCT, but has been associated with some severe side effects. Although pilot gene therapy studies using viral vector in a XHIM mouse model showed the correction of the immune defect, treated mice developed abnormal lymphoproliferation due to unregulated gene expression from ectopic genomic loci.[135,136] Therefore, using gene editing tools to target the integration of the XHIM gene under the control of its endogenous promoter has become an optimal alternative approach for treatment of the disease. By using the TALEN, Hubbard et al. have first demonstrated the feasibility of this approach, achieved the restoration of normal expression of CD40L and the rescue of IgG class switching in XHIM patient T cells.[137] A later study by Kuo et al. developed TALEN and CRISPR/Cas9-based platforms to achieve site-specific integration of a human CD40L cDNA, into the 5′UTR of the gene allowing correction of all known disease-causing mutations in XHIM.[100] Both approaches were tested in T cells derived from XHIM patient first. Although the TALEN approach resulted basal CD40L expression in unstimulated edited cells, a 20% upregulated of CD40L was detected upon anti-hCD3/anti-hCD28 stimulation which is comparable to stimulated T cells from healthy donors. The corrected XHIM T cells demonstrated a normal receptor-binding activity to recombinant chimeric CD40-mulg. The data highlighted that a proportionally small number of gene-corrected T cells in XHIM may be sufficient to allow enough class-switching to ameliorate the disease. In CRISPR/Cas9 treated XHIM T cells, a high rate of targeted gene integration was attained with the restoration of physiologically-regulated CD40L expression and function. In targeting CD34 + cells from healthy donor, Kuo et al. have shown that both platforms gave rise to a similar level of allelic disruption rate in 8 biological replicates, 4 PBSC donors (29.1 ± 7.8% with TALEN, average 33% with CRISP/Cas9). A relative high targeted gene integration rate was observed in CRISP/Cas9 treated cells, particularly when gRNA and Cas9 protein were delivered as a RNP (to 20.8 ± 6.6%). Following transplantation of edited cells into NSG mice at 12–20 weeks, the targeted gene integration was detected in the bone marrow from 80% of mice, with integration rates ranged from 0.3% to 22%, a mean of 4.4% across all treatment groups. The analysis of thymi of engrafted mice showed that 60% of them had thymic reconstitution, with higher frequency of engraftment at 5 months post-transplantation compared to the 3 months. No off-target activity was detected based *in silico* predicted off-target sites for both TALENs and CRISPR/Cas9 in K562 edited cells. However, three off-target loci were observed when using IDLV capture approach in TALENS- edited K562 cell. High-throughput sequencing of off-target sites in HSPCs and K562 cell treated with TALENs mRNA demonstrated statistically significant gene disruption at one off-target site in HSPCs, and a different off-target site in both cells. However, there was no off-target site identified in CRISPR/Cas9 treaded cells using GUIDE-seq, which has a higher sensitivity for off-target detection.[138] Overall, the CRISPR/Cas9-based approach showed some advantages over TALENs in targeting integration of XHIM gene. This study paves an important step toward the development of a viable therapy for XHIM through site-specific gene correction.

The major hurdles in HSPCs gene editing

Although genome editing holds tremendous promise for the development of novel gene therapies, its application to HSPCs is still in its infancy, and many issues regarding this new technology need to be addressed before translating it into a safe clinical application. One major hurdle is the low efficiency, particular following transplantation of edited cells *in vivo*, where the engrafted cells and frequency of edited cells decline significantly within 8–12 weeks after transplantation, and continuously decline in prolonged period. This suggests that long-term repopulating stem cells either have failed to undergo genome editing due to their quiescence and more resistance to HDR, or they have been damage by the exposure to nucleases and have lost their ability to self-renew. The high rate of indels rate generated *in vitro* in HSPCs may not necessary lead to sufficient level of HDR due to a significant activation of the P53 pathway which inevitably results in cell cycle arrest.[139,140] Haapaniemi et al. have recently demonstrated that the inhibition of p53 can prevent DNA damage response and increase HDR in immobilized human retinal pigment epithelial cells.[139] Whether the approach could be beneficial in editing HSPCs is yet to be tested. However, it is plausible that inhibition of P53 in HSPCs may lead to reduced protection of their integrity which could lead to transformation of the cells. Therefore, the approach needs to be carefully assessed. In addition, selective editing of sorted primitive HSPCs may present a strategy to ensure that only those long lived cells are adequately targeted. Resolving these biological and technical issues is crucial to move the field forward to a clinical therapy.

The off-target cutting via engineered nucleases is another concern for the clinical application of HSPC gene editing.[141] The genotoxicity generated from off-targets in HSPCs will pass to their progeny resulting in more profound effects than other tissue types. Therefore, the off-targets events should be defined and reduced to the minimum before genome editing can be translated into a safe clinical application. The RNA-guided CRISPR-Cas9 is likely to cause more unintended off target compared to ZFNs and TALENs due to its relatively short binding base-pairing.[141] Some commonly used methods for off-target detection have been discussed in this review. There are more methods have also been developed particular for CRISPR-Cas9.[82,142] However, each method has different strengths and weaknesses, and there is no single method is currently expected to be definitive or comprehensive for detecting mutations with

frequencies <0.1% in a cell population. Therefore, until an ideal method is developed, using combined methods is likely to give increased reliability. Off-target events can be reduced by increasing the specificity for the on-target. This can be achieved by modifying nucleases,[102,143,144] or using alternative class of the CRISPR system, such as CRISPR/Cpf1.[145] However, most of these approaches have not been applied to HSPCs yet, with the exception of a very recent study from Vakulskas et al.[102] Using a newly develop HiFi-Cas9, the authors achieved a high on-target activity while reducing off-target editing in HSPCs. Moreover, they also demonstrated that HiFi Cas9 is capable to mediate high level correction of the SCD causing mutation (p.E6V mutation) in HSPCs derived from SCD patients.

Apart from above, currently almost all gene editing studies carried out in HSPCs have been conducted in a relative small scale with a very limited number of patient samples being tested. From which the safety and efficacy profiles cannot be established. Therefore, significant effort has to be made on vigorous preclinical safety and tumorigenicity study in a large clinical relevant scale. Despite many roadblocks ahead of gene editing field, as researchers in the field continue to focus on the technical and biological challenges of increasing gene integration in long-term repopulating HSPCs, it is expected that improved outcomes will ultimately lead to clinical trials.

Conflict of interest

The authors declare no conflict of interest.

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