**Hematopoietic stem cell gene editing and expansion: state-of-the-art technologies and recent applications**

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**Keywords**

Hematopoietic stem cell, transplantation; gene editing, *ex vivo* expansion, HSCT

**Highlights**

* Review of hematopoietic stem cell (HSC) transplantation (HSCT) therapies
* Discusses recent progress in HSC gene editing using Cas9-AAV6
* Summarizes recent developments in *ex vivo* expansion using UM171

**Abstract**

Hematopoietic stem cell transplantation (HSCT) is a curative therapy for a range of hematological diseases, from leukemias to immunodeficiencies and anemias. HSCT aims to replace a patient’s dysfunctional blood system with a functional one by transplanting healthy hematopoietic stem cells (HSCs). HSCs may be collected from a healthy donor (for allogeneic HSCT) or from the patient for genetic correction (for autologous HSCT gene therapies). Despite the curative potential of HSCT, several hurdles to their wider and safer use remain, including how to efficiently genetically correct HSCs and how to increase donor HSC numbers to improve the donor pool. In recent years, the development of state-of-the-art technologies, such as Cas9-AAV6 technologies and identification of the small molecule HSC agonist UM171, have accelerated progress in HSC gene editing and expansion. These translational research efforts were the focus of the Spring 2021 International Society for Experimental Hematology (ISEH) webinar. Here we present a summary and discussion of the implications of these new approaches to improve HSC-based therapy.

**Introduction**

Hematopoietic stem cells (HSCs) are a rare bone marrow (BM) population of cells that are multipotent and able to self-renew, and can therefore stably reconstitute the entire hematopoietic system following transplantation [[1](#_ENREF_1), [2](#_ENREF_2)]. This concept forms the basis for HSC transplantation (HSCT) therapies. These currently involve ablating a patient’s dysfunctional hematopoietic system by radiation/chemotherapy and subsequent transplantation of healthy donor HSCs to reform a functional hematopoietic system within the patient. HSCT is used clinically for a wide range of blood disorders, from leukemias to immunodeficiencies and anemias, and still represents the only curative treatment option for many hematological diseases [[3](#_ENREF_3), [4](#_ENREF_4)].

Allogeneic HSCT is the most common form, in which HSCs from a healthy donor are collected from BM or peripheral blood (following stem cell mobilization) for transplantation into a suitably immune-matched patient [[3](#_ENREF_3)]. Immune matching is critical for allogeneic HSCT because a major adverse effect of this therapy is graft-vs-host disease (GvHD), caused by allo-reactive donor lymphocytes transplanted alongside donor HSCs that attack the recipient patient host’s tissues, resulting in morbidity and mortality. In addition, immunologic rejection of the incoming allogeneic cells is more frequent and harder to prevent with the greater degree of human leukocyte antigen (HLA) mismatch. An alternative source of donor HSCs is umbilical cord blood (CB), which is associated with reduced risk of GvHD due to the differing composition of the graft [[5](#_ENREF_5)]. However, the low number of HSCs that can be isolated from CB often limits the availability of this option; if insufficient numbers of donor HSCs are transplanted, graft failure can occur. Unfortunately, these restrictions and associated risks currently curb the availability of this potentially curative therapy.

The potential of gene therapy to treat monogenic disorders was formally recognized about 50 years ago, given that many diseases could be traceable to a specific malfunctioning gene. It was proposed that one could take stem cells from a patient and genetically modify them so that a normal protein could be made in place of (or alongside) an aberrant one when transplanted back to the individual [[6](#_ENREF_6)]. Despite the simplicity of this concept, it is only over the last decade that the field has started to implement the visions described in the 1970’s and we are now seeing some real success in the commercialization of gene therapy products.

Autologous HSCT gene therapies are now being developed and used as an alternative to allogeneic HSCT to treat several inherited blood diseases, including immunodeficiencies and anemias [[7](#_ENREF_7), [8](#_ENREF_8)]. Return of genetically corrected HSCs to the patient results in the formation of a corrected hematopoietic system and, importantly, avoids the potential for GvHD. However, a major issue in the field has been how to safely and effectively genetically modify these cells without the creation of off-target genomic instability that could lead to cancer causing mutations [[9](#_ENREF_9)].

In March 2021, the International Society for Experimental Hematology (ISEH) New Investigator Committee held a webinar focused on recent progress in HSCT therapies. This included presentations from Prof. Matthew Porteus (Stanford University), on the development of Cas9-adenoassociated virus (AAV)6 technologies for efficient HSC gene editing, and Prof. Guy Sauvageau (Université de Montréal), on the development of UM171-based *ex vivo* HSC expansion methodologies. Here, we summarize the recent research progress covered within this webinar (also available to view online at <https://www.iseh.org/page/ISEHWebinars>) and discuss the implications of these new approaches to improve HSC-based therapy.

**Applications of Cas9-AAV6 technologies for HSC gene editing**

The first demonstration that one could modify the genome of a somatic cell in a targeted way came about in 1985 [[10](#_ENREF_10)] but until 1994 the efficiency of gene editing in a mammalian cell was ~1 in a million [[11](#_ENREF_11)]. This pioneering work demonstrated that the generation of a DNA double-strand break as a recognition site in a target gene could stimulate the targeted integration process by a factor of 1000 in somatic cells. The foundational principle of the field of genome editing was established: prompting DNA damage could, in turn, induce DNA repair.

A major challenge with previous systems was their use of a specific homing endonuclease (an enzyme that recognizes and cuts at an exact DNA sequence) that was not amenable to re-engineering to recognize novel target sites, thus could not be applied to human cells. It is here that Prof. Porteus began his journey into the field, by driving the development of designer nucleases that could recognize target sites in endogenous genes and stimulate genome editing at those locations [[12](#_ENREF_12)]. Specifically, the development of zinc-finger nucleases, in which a DNA-binding protein with a defined recognition sequence is fused to a non-specific nuclease domain, allowed stimulation of the gene correction process as efficiently as the I-SceI homing endonucleases. In parallel, it had been shown that these enzymes not only prompt a gene correction process but could also be a way of generating mutations at the site of the break that could lead to repair by non-homologous end joining or homologous recombination [[13](#_ENREF_13)].

CRISPR-Cas9 has transformed the field of genome editing, facilitating the formation of active nucleases with high activity in a wide variety of cell types and with remarkable specificity [[14](#_ENREF_14)]. The group of Prof. Porteus focused their efforts on defining the key requirements for efficient *ex vivo* genome editing in cells. It was shown that one must introduce a high quantity of the nuclease and donor template to the cells in order to bias genetic repair towards homologous recombination [[15](#_ENREF_15)]. It is also important to work with a population of cells that is healthy and actively cycling given that homologous recombination-based editing is specifically enhanced by cellular proliferation, particularly in human CD34+ hematopoietic stem and progenitor cells (HSPCs) [[16](#_ENREF_16)].

Primary human cells have sensing mechanisms for foreign nucleic acids, which are essential for protection against viral infections. This hinders the use of DNA plasmids to deliver the components required for *ex vivo* HSC editing, as they trigger a type 1 interferon response in these cells, halting cellular proliferation, protein synthesis and ultimately driving cell death [[17](#_ENREF_17)]. With this in mind, the Porteus group established a gene editing system that combines CRISPR-Cas9 technology with the use of recombinant adeno-associated viruses (AAV) [[18](#_ENREF_18)]. After purifying a cell population of interest (e.g., CD34+ HSPCs) and maintaining it in culture conditions that promote cell cycling, the CRISPR-Cas9 nuclease is delivered as a stable ribonuclease protein (RNP) complex by electroporation. The donor vector is provided directly after making use of the AAV6 serotype, which was found to transfer high levels of cargo to the nucleus, avoiding detection by cytoplasmic sensors and with minimal transcriptional perturbation to the cells themselves [[17](#_ENREF_17)].

This technique for gene correction in human HSPCs promised to be a safe and effective therapy for a range of genetic diseases. Specifically, the monogenic disorder sickle cell disease (SCD) proved a logical target as it is caused by a single point mutation in the β-globin gene that results in the production of sickle hemoglobin and ultimately impairs red blood cell (RBC) function. The only curative therapy available for this condition is allogeneic HSCT from a matched donor [[19](#_ENREF_19)]. As one of the most common genetic diseases in the world and given the challenges of allogenic transplantation, the advent of Cas9-AAV6 technologies opened the potential to replace allogenic HSCT with transplantation of corrected autologous HSPCs – allowing each patient to be their own donor, thus eliminating immune complications that often arise. Using this system, Prof. Porteus and colleagues demonstrated 60-80% of sickle allele correction in CD34+ cells cultured *ex vivo* [[20](#_ENREF_20)] and when differentiated *in vitro* to generate RBCs, pathologic hemoglobin (Hgb) S was converted to non-pathological HgbA at significantly high levels. The rapid advancement of this gene correction technology as a potentially curative therapy for SCD is promising, however many challenges remain including the need for high editing efficiency and ensuring low off-target effects.

In order to assess the long-term engraftment potential of *ex vivo* gene corrected human HSPCs, since the durability of an autologous HSCT depends on the ability to modify HSCs permanently, most preclinical studies have utilized xenograft transplantation into an immunodeficient mouse model. However, these assays preclude the development of mature human RBCs, restricting the analysis of gene correction of erythropoietic defects to *in vitro* studies. To overcome this hurdle, Wilkinson and colleagues used Cas9-AAV6 technology to target the *Rosa26* locus in mouse HSCs and serial transplantation experiments confirmed that gene targeting of long-term HSCs was achieved [[21](#_ENREF_21)]. Having established this, they evaluated the functional consequences of HgbS gene correction in the Townes mouse model of SCD and were able to demonstrate that this improves *in vivo* RBC indices following autologous transplantation. Altogether, these advances in the field provide support for the clinical investigation of Cas9-AAV6 gene correction therapies and phase I/II clinical trials on gene correction for SCD patients are in the pipeline.

A further, recent, application of the combined Cas9-AAV6 genome editing method was on HSPCs from β-thalassemia patients [[22](#_ENREF_22)]. These individuals suffer from severe hemolytic anemia due to the loss of β-globin (HBB) as well as erythrotoxic accumulation and aggregation of the β-globin binding partner, α-globin (HBA1/2). As with SCD, currently the only curative strategy is allogenic HSCT but ideally treatment would involve the isolation of patient derived HSPCs and the introduction of *HBB* to restore hemoglobin production, followed by autologous HSCT of the patient’s own corrected HSPCs. By leveraging the Cas9-AAV6 gene correction method, the entire *HBA1* gene could be replaced with a full length *HBB* gene in β-thalassemia-derived HSPCs, which was sufficient to normalize the β-globin: α-globin protein ratios and restore functional hemoglobin tetramers in patient derived RBCs. These HSPCs were capable of long-term reconstitution in mice, establishing proof of concept for the replacement of HBA1 with HBB as a novel therapeutic strategy for curing β-thalassemia [[22](#_ENREF_22)].

**UM171 in HSC expansion and rejuvenation**

One of the requirements to perform *ex vivo* HSC gene editing is the ability to maintain HSCs undergoing editing in *ex vivo* culture so that functional HSCs can be returned to the patient and stably reconstitute hematopoiesis. Development of culture conditions that stably maintain and expand functional HSCs has been a major challenge in the field. *Ex vivo* HSC expansion also holds much promise to improve HSCT more generally by improving HSC dose for transplant and patient access to CB-derived HSCs [[2](#_ENREF_2), [23](#_ENREF_23)]. Overcoming this barrier has been a long-term aim of Prof. Guy Sauvageau’s research group and was summarized in his webinar presentation.

In the context of autologous HSCT gene therapies, two recent clinical trials that evaluated *BCL11A* modulation to improve SCD have highlighted the importance of improving cell potency during *ex vivo* manipulation [[24](#_ENREF_24), [25](#_ENREF_25)]. One approach used lentivirus-based shRNA knockdown of *BCL11A* [[24](#_ENREF_24)] while the second used CRISPR-based mutation of a *BCL11A* enhancer [[25](#_ENREF_25)]. In both cases, the aim was to reactivate fetal hemoglobin and thereby rescue the defects of the mutant form of adult hemoglobin in SCD patients. While both methods showed promising results, these clinical trials needed to use high donor HSC doses (up to 1.6x107 CD34+ cells/patient kg). Additionally, even with these large cell doses, neutrophil reconstitution was delayed with engraftment taking 20-33 days. Without neutrophils, patients are vulnerable to infection and thus time to neutrophil engraftment is a major risk factor for HSCT [[4](#_ENREF_4)].

Traditional media conditions (containing hematopoietic cytokines and serum albumin) that support proliferation of mature blood cells rapidly leads to loss of functional HSCs [[23](#_ENREF_23), [26](#_ENREF_26)]. Over the years, various approaches have been suggested to improve HSC maintenance and stimulate *ex vivo* self-renewal. These include small molecules or recombinant proteins such as dmPEG2 [[27](#_ENREF_27)], LSD1 inhibitors [[28](#_ENREF_28)], HDAC inhibitors [[29](#_ENREF_29)], nicotinamide [[30](#_ENREF_30)], SR1 [[31](#_ENREF_31)], and GNFR [[32](#_ENREF_32)] as well as modifications to the base culture conditions via use of zwitterionic hydrogels [[33](#_ENREF_33)] or polyvinyl alcohol [[34](#_ENREF_34)]. From a small molecule screen, the Sauvageau lab identified UM171 as an HSC agonist [[35](#_ENREF_35)], which has become a widely used experimental tool. In their efforts to quantify functional HSCs *ex vivo*, the Sauvageau lab have also validated several *ex vivo* HSC markers including CD201 [[36](#_ENREF_36)] and integrin-α3 (ITGA3) [[37](#_ENREF_37)]. This is important because surface markers used to isolate fresh HSCs are not always conserved after culture (e.g., CD38 and CD49f no longer help to resolve HSCs *ex vivo*).

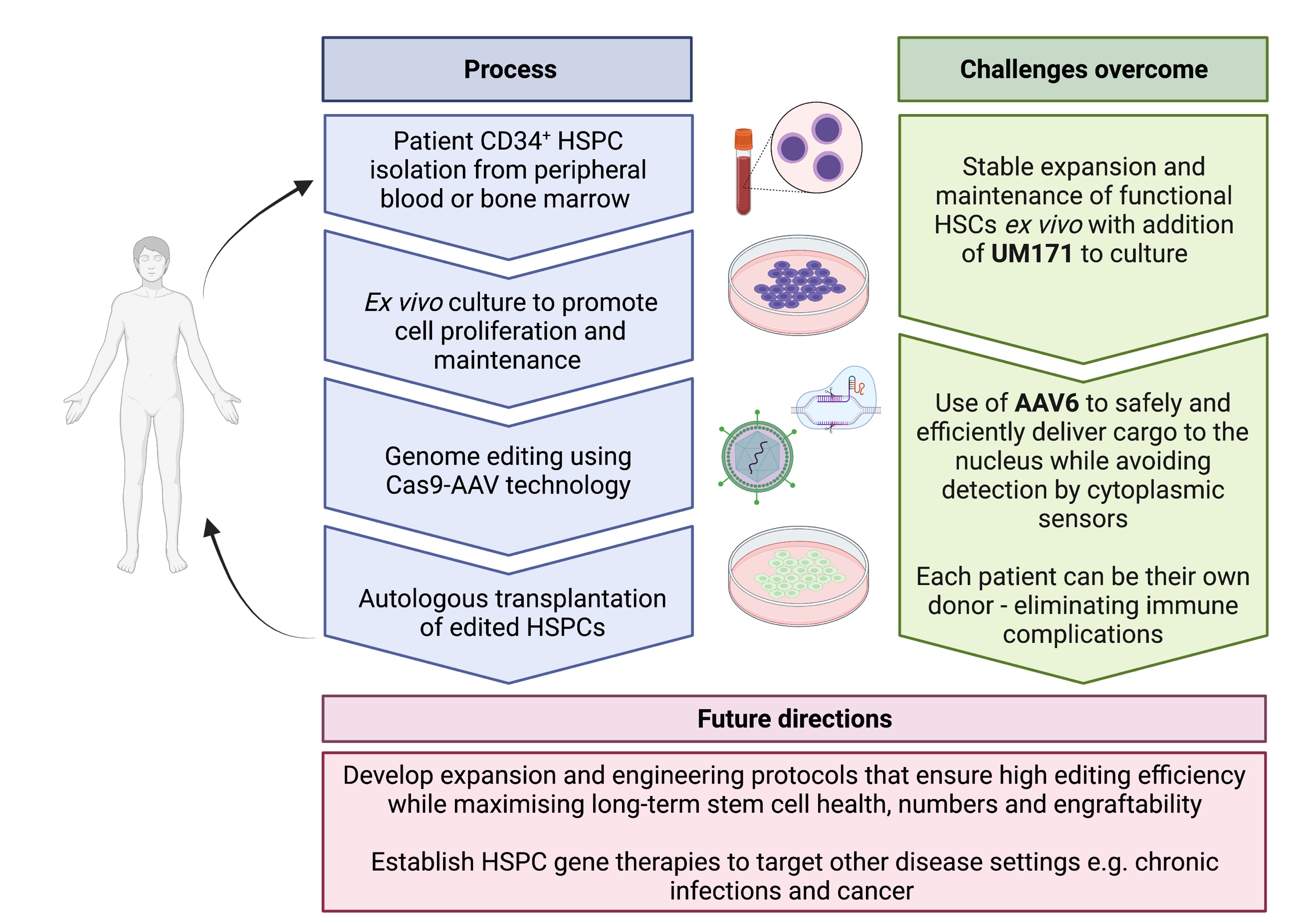
Since identification of UM171 in 2014, UM171 expanded CB has been evaluated to a phase I/II allogeneic HSCT clinical trial published in 2020 [[38](#_ENREF_38)], where it was found to be safe and also reduced transplant related mortality and GvHD. An impressive 7-day, 50-fold expansion of CD34+ HSPCs can now be achieved using clinical-grade methods. However, the underlying mechanism of action has long been missing. The Sauvageau lab has recently identified several mechanisms of UM171 activity, which help to explain its mechanism of action. Initial work by the Sauvageau lab identified a role for UM171-mediated HSC expansion in limiting reactive oxygen species (ROS) levels by reducing mitochondrial metabolism and activating pro-inflammatory signaling to limit ROS levels [[39](#_ENREF_39)]. However, the exact target of UM171 remained elusive.

In characterizing CD34+ HSPCs cultured with or without UM171, the Sauvageau lab identified global loss of histone 3 lysine 4 di-methylation (H3K4me2) and histone 3 lysine 27 acetylation (H3K27ac) in culture [[40](#_ENREF_40)]. However, UM171 rescued this effect of culture and sustained H3K4me2/H3K27ac levels. Through genetic screening, KBTB4 was identified as the target of UM171, which was required for UM171-mediated HSC expansion. BioID experiments identified KBTB4 as part of the CRL3 E3 ubiquitin ligase complex and also interactions with RCOR1 and LSD1 (KDM1A), which function as part of the demethylase/deacetylase REST complex. Further work demonstrated that UM171-mediated recruitment of CRL3 to RCOR1/LSD1 led to their degradation, helping to explain how UM171 prevents loss of H3K4me2/H3K27ac in HSCs *ex vivo* [[40](#_ENREF_40)].

These results complemented similar work from the Larsson lab, who discovered that LSD1 inhibitor was downstream of UM171 and that LSD1 inhibitors partially mimicked the UM171 phenotype [[28](#_ENREF_28)]. However, differences between UM171 and LSD1 inhibitors remain, likely due to the different modes of action. LSD1 is a multifaceted protein [[41](#_ENREF_41)], acting as both a histone demethylase as well as a scaffold protein for E3 ubiquitin ligases, which targets proteins for degradation. LSD1 inhibitors block demethylase activity while UM171 acts as a LSD1 degrader and therefore inhibits both activities [[40](#_ENREF_40)]. Together, these studies highlight the potential of *ex vivo* HSC expansion to improve HSCT and the identification of the UM171 opens up prospects to identifying novel HSC expansion agonists. In the future, it will also be interesting to consider what normally sustains H3K4me2/H3K27ac levels in HSCs in vivo, and investigate how *ex vivo* HSC expansion mechanisms relate to the *in vivo* counterpart.

**Conclusions**

Recent progress in HSC gene editing and *ex vivo* expansion have opened up exciting new opportunities to cure a range of hematological diseases (Figure 1). Further development of these technologies and exploring how synergy between them may offer novel opportunities for treating human disease remain promising lines of research. For example, stable HSC expansion methods could allow for more sophisticated HSC engineering protocols to be performed – not only to correct genetic mutations but also to confer new cellular potential. Given that both *ex vivo* culture and genetic manipulation are unnatural and stressful, a major goal for the field is to develop protocols that maximize long-term stem cell health, numbers and engraftability. Defining the characteristics of healthy stem cells and how to achieve and maintain these *ex vivo* remains an important area of investigation. The hemoglobinopathies discussed here have severe effects, not only on patients themselves, but their families and wider communities also. We still face many research, scientific and justice challenges to bring these therapies to patients all over the world. However, given the rapid progress in these fields just over the last decade, it is exciting to think about the prospects for these next-generation HSC therapies and how they may mitigate the impacts of disease on individuals, families and communities alike.



**Figure 1:** Recent advances and future directions for HSC editing and expansion.Autologous HSPCs are collected from the patient and enriched for CD34+ cells cultured in the presence of cytokines and small molecules before genetic modification using Cas9-AAV technology. After conditioning the patient to deplete endogenous HSPCs, the edited cells are transplanted back. Recent efforts to develop protocols and technology have allowed the field to overcome technical challenges and improve HSC gene editing and expansion efficiency. Future work to improve these methodologies will offer novel opportunities for treating human disease.

**Acknowledgements**

We thank Prof. Guy Sauvageau for presenting at the ISEH webinar, and ISEH staff and New Investigator Committee for their support. M.H acknowledges funding from the Wellcome Trust. This research was funded in part by the Wellcome Trust [203151/Z/15/Z] and the UKRI Medical Research Council [MC\_PC\_17230]. For the purpose of open access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission. A.C.W acknowledges funding from the Kay Kendall Leukaemia Fund. M.H.P. gratefully acknowledges the support of the Amon Carter Foundation, the Laurie Kraus Lacob Faculty Scholar Award in Pediatric Translational Research and the NIH (R01AI097320; R01AI120766). Figure created with BioRender.com.

**CompetingInterests**

M.H.P. has equity and serves on the scientific advisory board of CRISPR Therapeutics and Allogene Therapeutics. However, none of these companies had input into the design, execution, interpretation, or publication of the work in this manuscript. All other authors do not declare any competing interests.

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