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***In vivo* hematopoietic stem cell transduction**

Maximilian Richter^{1,#}, Daniel Stone^{6,#}, Carol Miao^{5,8}, Olivier Humbert⁷, Hans-Peter Kiem^{3,7}, Thalia Papayannopoulou², and Andre Lieber^{1,4,*}

¹Division of Medical Genetics, University of Washington, Seattle, WA

²Division of Hematology, University of Washington, Seattle, WA

³Department of Medicine, University of Washington, Seattle, WA

⁴Department of Pathology, University of Washington, Seattle, WA

⁵Department of Pediatrics, University of Washington, Seattle, WA

⁶Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA

⁷Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA

⁸Seattle Children's Research Institute, Seattle, WA

Synopsis

Current hematopoietic stem cell (HSC) gene therapy approaches rely on the collection of patient HSCs, followed by their culture and expansion *in vitro*, their modification using γ -retrovirus or lentiviral vectors, and their re-infusion into myelo-conditioned patients. While this approach has been successfully used in numerous clinical trials, its reliance on the extended *ex vivo* culture of patient HSCs comes with a set of disadvantages. Culturing HSCs in the presence of a cytokine cocktail to facilitate their expansion and transduction is thought to negatively impact the long-term viability of HSCs, and their homing and repopulation capacity. Importantly, the requirement for myeloablative regimens in patients represents a critical risk factor and results in considerable morbidity. In addition to these biological problems, the process of *ex vivo* HSC manipulation is also challenging from a logistics and regulatory standpoint, as manipulations must be performed in specialized, accredited centers. These complexities lead to a high price of such gene therapeutic regimens, and subsequently severely limited patient access to these treatments. *In vivo* HSC gene therapy approaches aim to simplify the gene therapy process by eliminating the need for *ex vivo* handling of patient HSCs. The *in vivo* approach can be subdivided into three methods. One method is based on the direct modification of HSCs in the bone marrow following intraosseous injection. Another involves intravenous injection of gene delivery vectors that home to HSCs in bone marrow. We have developed an alternative approach in which HSCs from the bone marrow

*corresponding author: Andre Lieber, Division of Medical Genetics, University of Washington, Box 357720, Seattle, WA 98195, lieber00@uw.edu, phone: (206) 221-3973.

#equal contribution

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are mobilized into peripheral blood, become gene modified upon intravenous vector administration, and then re-engraft in the bone marrow. We will provide examples of these three approaches and then discuss the advantages and disadvantages of *in vivo* HSC gene therapy.

Keywords

intravenous; intraosseal; viral vectors; mobilization

Introduction

HSCs in the bone marrow

The majority of HSCs reside in so-called stem cell niches regions of the bone marrow in which non-hematopoietic cells interact with HSCs and regulate their dormancy and self-renewal or their differentiation and expansion¹. However, a small fraction of HSCs circulate in the peripheral blood under steady state conditions². HSCs that leave the bone marrow under steady state conditions provide a means of exchange between different stem cell niches as well as a way to react to local damage within hematopoietic tissues. Even under normal conditions, circulating HSCs appear to be able to rapidly re-engraft in bone marrow so that there is a cycle of constant egress and re-engraftment of HSCs³. HSC regulation and retention within the bone marrow stem cell niche is mediated through multiple interactions between HSC surface receptors and their respective ligands expressed or secreted by surrounding cells, including osteoblasts, sinusoidal endothelial and perivascular cells.

HSC mobilization

The induced egress of HSCs is referred to as stem cell mobilization. While egress of HSCs from the bone marrow can be observed in response to stress, for example following injury of hematopoietic organs, stimulated mobilization is commonly achieved through drug administration. The most commonly used mobilizing agent is recombinant human G-CSF, which is given in the form of subcutaneous injections for 5 days, and leads to efficient mobilization of both HSCs as well as more differentiated cells². Another class of mobilizing agents are CXCR4 antagonists, most prominently the FDA-approved drug Plerixafor/AMD3100. CXCR4 antagonists lead to more rapid mobilization of HSCs than G-CSF⁴, and are thought to cause mobilization solely through disruption of the SDF-1-CXCR4 axis. AMD3100 has been shown to synergize with G-CSF mobilization. Due to its higher mobilization power, the combination of G-CSF and AMD3100 is used as a regimen in poor HSC mobilizers such as chemotherapy patients⁵. In a similar manner soluble SCF is able to interrupt the connection between membrane-bound SCF and c-kit, even though SCF is considered to be a slow mobilizing agent and has to be administered for several days⁶. Another class of mobilizing agents target VLA-4, and both VLA-4 binding antibodies⁷ and small molecules⁸ are able to rapidly mobilize HSCs. VLA-4 binding agents are also thought to have a synergistic or additive effect when used together with G-CSF and AMD3100⁹. One VLA-4 inhibitor, the small molecule BIO5192, was shown to efficiently and rapidly mobilize HSCs. Furthermore, BIO5192 can be combined with G-CSF alone or with AMD3100 to increase levels of mobilized HSCs. BOP (N-(benzenesulfonyl)-L-prolyl-L-O-

(1-pyrrolidinylcarbonyl)tyrosine), a small molecule targeting $\alpha 9\beta 1/\alpha 4\beta 1$ integrins also rapidly mobilizes long-term multi-lineage reconstituting HSC¹⁰. Synergistic engraftment augmentation is observed when BOP is co-administered with AMD3100.

Examples for *in vivo* HSC gene therapy (Fig. 1)

Example 1: Intravenous injection of triplex-forming peptide nucleic acids (PNAs)

PNAs are designed to bind site-specifically to genomic DNA via strand invasion and formation of PNA/DNA/PNA triplexes with a displaced DNA strand. PNAs consist of a charge-neutral peptide-like backbone and nucleobases enabling high affinity hybridization with DNA. PNA/DNA/PNA triplexes can be used to modify DNA by recruiting endogenous DNA repair proteins to initiate site-specific modification of the genome when single-stranded 'donor DNAs' are co-delivered as templates containing the desired sequence modification¹¹.

A recent study reported *in vivo* HSC gene editing in mice using intravenously injected triplex-forming PNAs in combination with SCF given i.p. 3 hours before the PNAs¹². Treatment of transgenic mice carrying a β -globin/GFP reporter transgene with PNAs and single-stranded donor DNA yielded gene editing in mouse CD117⁺ cells at frequencies up to 1% with a single treatment. In a thalassemic mouse model, *in vivo* treatment resulted in a gene editing frequency of almost 4% in total bone marrow mononuclear cells and 6.9% in HSCs, resulting in amelioration of the disease phenotype. The authors reported that, based on their *ex vivo* studies, SCF could enhance PNA-mediated gene editing *in vivo*. We speculate that SCF treatment might also have resulted in HSC mobilization and thus caused better gene transfer. A non-viral PNA-based platform for *in vivo* HSC gene editing has the advantage of circumventing host immune responses and, if it can be scaled-up, has great potential over designer nucleases-based approaches for gene editing that introduce an active nuclease into cells, which can lead to off-target cleavage in the genome and can affect the viability and function of HSCs.

Example 2: Intravenous injection of foamy virus vectors in dogs

Current VSVG-pseudotyped lentivirus vectors are not suitable for direct *in vivo* injection, since VSVG is rapidly inactivated by human complement¹³. In this respect, several pseudotyping partners have emerged in the field, such as the complement-resistant gibbon ape leukemia virus (GaLV) or the endogenous feline leukemia virus RD114 glycoproteins. The Kiem group has used foamy virus (FV) vectors, which are non-pathogenic integrating retroviruses with properties that distinguish them from other viral vectors. Unlike VSV-G pseudotyped lentiviral vectors, FV vectors are resistant to human serum inactivation¹⁴, which gives them a specific advantage in the context of *in vivo* delivery. FV vector integrations are less frequent near transcriptional start sites when compared to γ -retrovirus vectors, and FV vectors also do not show a preference for integrating within highly expressed genes, as has been observed for lentivirus vectors¹⁵. Importantly, Hendrie *et al.* also showed that FV vector proviruses are less likely to activate nearby genes even if they do integrate close to them¹⁶. A number of groups demonstrated that FV vectors are highly proficient in gene transfer to HSCs from both human and large animals, such that promising

therapeutic constructs can be efficiently evaluated under preclinical settings. Safety of FV vectors for gene therapy applications has also been established in dogs¹⁷ and in human SCID repopulating cells¹⁸. FV vectors thus offer a unique combination of properties that makes them a potentially superior choice for retrovirus gene therapy and for therapeutic gene corrections with diseases such as SCID-X1. We have used the canine SCID-X1 model, which is a naturally occurring genetic disease caused by inactivating mutations in the IL2RG gene that encodes the shared common gamma chain (γ C) signaling component of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors¹⁹. Canine SCID-X1 provides an excellent pre-clinical model as it manifests nearly identical characteristics compared to human SCID-X1. Both disorders are characterized by absent thymic T-cell development leading to an absence of mature peripheral T-cells, resulting in the lack of T-cell mediated immune responses and dysregulated B cell germinal center responses that, in turn, lead to low IgA, IgM and IgG levels, absence of lymph nodes, failure to thrive, and early infant mortality due to viral and/or bacterial infection²⁰.

We and others have explored *in vivo* gene therapy²¹. In collaboration with Dr. Peter Felsburg we have investigated *in vivo* delivery using FV vectors in SCID-X1 dogs. Intravenous injection of a FV vector expressing a codon-optimized human γ C gene in newborn SCID-X1 dogs resulted in expansion of corrected CD3 T lymphocytes that expressed the CD4 or CD8 co-receptors, underwent antigen receptor gene rearrangement, and were functionally mature enough to respond to T cell mitogens. Furthermore, FV integration site analysis demonstrated that the provirus did not integrate near known proto-oncogenes, and there was no evidence of clonal expansion that indicated a leukemic transformation²¹. We also evaluated generation of specific antibody responses and immunoglobulin class-switching in treated animals after immunization with the T cell dependent neoantigen bacteriophage, phiX174. This neoantigen has been used to evaluate dogs and humans with SCID-X1 before and after bone marrow transplantation and gene therapy. We found that treated animals showed a primary and secondary antibody response that is very similar to that seen in normal human controls, indicating that our treatment restored both the B and T cell cytokine signaling that is required for class switching and memory responses to this neoantigen. Altogether, our results demonstrate that *in vivo* gene therapy using FV vectors is safe. *In vivo* delivery of γ C expressing FV vector to dogs resulted in immune reconstitution with gene-corrected T-cells but the treated animals still developed infections and had low immunoglobulin levels and marking levels in granulocytes and monocytes were very low (0.6%), indicating that further increase in the efficacy of HSC transduction is required to achieve long-term phenotypic correction. There are several ways in which this type of *in vivo* gene therapy can be further optimized. The kinetics of immune reconstitution may be enhanced by modifying FV vector design, for example, by using a stronger promoter in place of the currently used human elongation factor-1 alpha promoter (EF1 α) to drive expression of γ C. In addition, gene marking in other cell lineages that do not have a selective advantage like T lymphocytes may be increased by more efficiently targeting HSCs. This could be accomplished by using mobilizing agents to increase the number of circulating HSCs in peripheral blood or by the *in vivo* selection of gene-modified HSCs. Importantly, the *in vivo* gene therapy procedure developed here for SCID-X1 is

highly portable and could be disseminated worldwide for the treatment of additional genetic blood disorders.

Example 3: Intraosseal lentivirus injection in mice

Although lentiviral vectors (LV) have traditionally been used in *ex vivo* HSC gene therapy, recent advances showed that LV can have utility for *in vivo* gene therapy. As an example, we will discuss LV-mediated FVIII gene transfer by intra-femoral injection in mice. LV-mediated gene transfer into HSCs resulted in stable integration of FVIII gene into the host genome, leading to a persistent therapeutic effect^{22–24}. This IO infusion method was improved by using a syringe pump to slowly infuse LVs into the bone marrow so that more vectors can be retained for longer in the marrow cavity in order to achieve high levels of transduction of bone marrow cells²². In another study, a single infusion of GFP-LV achieved persistent GFP expression in 10–50% of HSCs for up to 160 days, indicating that IO LV delivery mediates sustained transduction of hematopoietic stem/progenitor cells^{22,25}. Furthermore, IO delivery of E-F8-LVs encoding the FVIII gene driven by a ubiquitous EF1 α promoter produced FVIII expression in various types of cells including antigen presenting cells, and high levels of FVIII were initially secreted into the circulation. However, a high-titer inhibitory antibody response to FVIII was rapidly induced, which completely eliminated functional FVIII activity. In contrast, a single IO infusion of G-F8-LVs driven by a megakaryocyte-specific promoter produced long-term stable expression of FVIII in platelets and initiated a long term corrected hemophilia phenotype²². Most importantly, this strategy was also proven successful in hemophilia A mice with pre-existing inhibitory antibodies²². This is because FVIII stored in α -granules of platelets is protected from high-titer anti-FVIII antibodies, and the locally excreted FVIII following platelet activation that participates directly in clot formation is functionally more potent than plasma FVIII.

Another example for IO injection was described by Frecha *et al.*¹³. They used LVs displaying stem cell factor and thrombopoietin, which allows for targeting of the vector particles to HSCs expressing c-Kit and c-Mpl. These vectors were more efficient in transducing CD34+ cells after intrafemoral injection into mice with engrafted human CD34+ cells. However, the overall percentage of GFP+ cells in human CD45+ cells in the BM was only 0.7%.

While IO LV injection in mice is technically difficult and challenging to standardize due to limited space for the injected virus in the bone, IO injection could be more feasible in large animals and humans. This is supported by studies with IO transplantation of HSCs. Intraosseous delivery of donor hematopoietic cells has been tested to mitigate the inevitable early loss of cells given I.V. to high blood volume organs, like liver and lung. It was first attempted in mice using different approaches^{26–28} and later in larger animals, canines²⁹, non-human primates³⁰ and cancer patients^{31,32}. The source of cells was bone marrow, cord blood, or mobilized peripheral blood and recipients were either myeloablated or received reduced intensity or no conditioning. Regarding the mouse, the overall assessment is that, despite multiple efforts, the outcome has not been better than the intravenous infusion, most likely because of anatomic constraints. In larger animals, the results differ depending on the number of cells used or the conditioning. The effort of using cord blood via the intraosseous

route is in its early stages and many variables influencing the outcome need to be firmly ascertained; these include the optimum volume to be injected, the cell concentration, the number of injection sites and the optimum conditioning.

Example 4: HSC transduction using adeno-associated virus vectors

Although we are not aware of published data describing direct *in vivo* HSC transduction following intravenous adeno-associated virus (AAV) vector delivery, this vector system has potential for *in vivo* HSC gene addition and targeting. Furthermore, AAV has proved highly promising in both preclinical and clinical safety and efficacy studies for hepatocyte targeted gene therapy^{33,34}. Several *ex vivo* studies with AAV and HSCs demonstrate their potential utility for *in vivo* HSC transduction, although HSCs from some but not all species can be efficiently transduced by AAV when the best serotype and culture conditions are used. AAV1 vectors, for example, can transduce mouse Lin⁻Sca1⁺Kit⁺ (LSK) cells, whereas CD34⁺ cells from cynomolgus monkeys are poorly transduced by AAV vectors derived from serotypes 1–10³⁵. A number of groups have shown that human HSCs can be readily transduced with AAV6 based vectors^{35,36}, and vectors with AAV6 capsids containing surface exposed tyrosine to phenylalanine mutations transduce human HSCs with the highest efficiency^{35,37}.

The ability of AAV vectors to efficiently deliver their genomes to the nucleus of HSCs has led to the development of gene targeting strategies, as their single stranded vector genomes can be efficiently used as donor templates during the process of homologous recombination directed targeted integration³⁸. Two recent studies demonstrated that co-delivery of an AAV6 donor template in combination with a target site directed endonuclease leads to efficient *ex vivo* gene editing in human HSCs that retain the ability to engraft in NSG mice and differentiate into multiple hematopoietic lineages³⁹. *In vivo* engraftment studies have also successfully been performed in mice using self-complementary AAV1 (scAAV) vector transduced LSK cells. Unlike classical AAV vectors, scAAV vectors do not require second strand synthesis or intermolecular annealing of their genomes to initiate transgene expression, so typically provide more efficient gene marking. Both primary and secondary transplant recipients showed up to 7% transgene expression in peripheral blood⁴⁰. More recently gene edited HSCs that were engineered using ZFN endonucleases and AAV6-derived targeting vectors showed long-term engraftment and differentiation in NSG mice⁴¹. A similar ZFN plus AAV6 based *ex vivo* approach to HSC gene editing has subsequently been used for the treatment of X-linked chronic granulomatous disease, with up to 11% of bone marrow cells in primary recipients containing an introduced copy of the gp91phox subunit of the NADPH oxidase⁴².

AAV does have some potential disadvantages for direct *in vivo* use such as the relatively high cost of production and limited knowledge of the *in vivo* tropism for AAV serotypes that can transduce HSCs *ex vivo*. For example, AAV6 shows high-affinity for skeletal muscle upon IV administration⁴³, which will likely impede attempts to target HSCs *in situ*. Furthermore, while efficient gene expression in the liver can theoretically be achieved from episomal AAV vector genomes, HSC gene therapy requires vector integration, a process that is not well studied in HSCs.

Example 5: *In vivo* transduction of primitive HSCs after mobilization and intravenous injection of integrating helper-dependent adenovirus vectors

Human adenovirus (Ad) has long been used as a gene transfer vector to various tissues. However, early studies showed that the most commonly used serotype, Ad5, was incapable of efficiently transducing HSCs. In contrast, other less studied Ad serotypes, especially those belonging to human species B, showed much more promise in their ability to transduce HSCs. A number of species of B Ads, including serotypes 11, 16, 21, 34, 35, and 50 use CD46 as a receptor⁴⁴, a membrane protein expressed on all nucleated cells in humans. Its main function is to protect self-tissue from inadvertent killing by the complement system, but it also has important signaling functions, for example in the regulation of T-cell activity⁴⁵. We and others have found that CD46 is uniformly expressed on all CD34+ cells^{44,46}.

In order to harness the well-understood biology of Ad5, the tools to manipulate and vectorize Ad, as well as the enhanced HSC transduction capabilities of species B Ads, fiber-chimeric vectors containing species B fibers on an Ad5 capsid were developed. Vectors carrying the fiber of CD46-tropic adenovirus type 35 (Ad5/35) were subsequently shown to efficiently transduce human HSCs^{46–50}. Importantly, after intravenous injection into mice and non-human primates, first-generation Ad5/35 vectors did not cause liver toxicity^{51–53}, a problem seen with serotype 5 (Ad5) based vectors⁵⁴. Moreover, our group introduced mutations in the Ad35 fiber knob to further increase its affinity toward the Ad35 cellular receptor CD46, leading to increased transduction capabilities when targeting HSCs⁵⁵.

To further increase the suitability as a gene transfer vector for HSCs, helper-dependent adenovirus vectors (HDAds) have been developed^{56–59}. These vectors are devoid of all viral genes and only contain minimal amounts of viral DNA. This completely abolishes the leaky expression of adenoviral genes that had been shown to lead to cytotoxicity in HSCs that have been transduced with earlier Ad vectors that still encoded these genes^{59,60}.

The goal of HSC gene therapy is the lifelong correction of an underlying genetic disease. To fulfill this requirement integration of the therapeutic transgene is generally required so that gene-corrected HSCs are able to give rise to gene corrected progeny cells. Ad5 however, does not actively integrate its genetic material into the genome of infected cells. To counteract this shortcoming, HDAd vectors can be armed with transposon-based transgene integration systems. Multiple Class II DNA transposons display activity in human cells, including Tol2, piggyBac, and Sleeping Beauty (SB). Tol2 and piggyBac have the propensity to integrate transposons in and around actively transcribed genes⁶¹. In our studies we have used a hyperactive Sleeping Beauty (SB100x) transposase system^{62,63}. The SB transposase, when co-expressed *in trans* from a second vector, recognizes specific DNA sequences (inverted repeats-“IRs”) flanking the transgene cassette and triggers its integration into TA dinucleotides of chromosomal DNA. Unlike retrovirus integration, SB-mediated integration does not depend on the transcriptional status of the targeted genes nor on cellular DNA repair proteins⁶⁴. Studies with human cell lines and mouse hepatocytes *in vitro* and *in vivo* showed that SB-mediated transgene integration is random and has not been associated with the activation of proto-oncogenes⁶³.

In our experience, intravenous injection of adenovirus vectors in mice and non-human primates does not lead to transduction of HSCs in the bone marrow, even if the vector is capable to efficiently transduce HSCs *in vitro*^{51,52}. We therefore decided to mobilize HSCs from the bone marrow and transduced them in the periphery with integrating HDAd5/35++ vectors.

In our studies, we employed the SB100x vector platform in the context of HSC gene therapy and used a human CD46 transgenic mouse model to study stable transfer a reporter gene cassette into HSCs *in vivo*⁶⁵. HSCs were mobilized from the bone marrow of mice into the peripheral blood through administration of G-CSF and AMD3100, after which integrating HDAd5/35 vectors were injected intravenously. We were able to show *in vivo* transduction following this regimen as reporter gene-positive LSK cells could be detected in the bone marrow of mice (Fig. 2A). We saw that 6.5% of bone marrow LSK cells expressed a reporter transgene at 20 weeks post *in vivo* transduction. Furthermore, HSCs transduced in this way could give rise to progenitor colonies consisting of reporter gene-positive progeny cells (Fig. 2B) and at 20 weeks after *in vivo* transduction 9% of CFU progenitor colonies were positive for the transgene. This showed that the reporter gene had been integrated into the HSC genome and that the gene modified cells were still functional stem cells capable of proliferation and differentiation. Importantly, CFU assays showed an increase in GFP-positive colonies over time indicating transduction of long-term surviving and potentially self-renewing cells capable of reconstitution of hematopoiesis in lethally irradiated transplant recipients. Moreover, we demonstrated that HSCs modified after *in vivo* transduction could rescue lethally irradiated mice upon transplantation and that the recipients of these transplants showed reporter gene expression in all hematopoietic lineages up to 16 weeks after transplantation. In addition, successful HSC transduction could be shown in a humanized mouse model when using a similar *in vivo* transduction regimen.

In addition to the general advantages of *in vivo* HSC gene therapy, the use of adenoviral vectors for this application provides a number of additional advantages. HDAd vectors have a relatively large cloning capacity of up to 34 kb. However, it has to be noted that when using the SB transposase, efficient transposition is only observed for cassettes of up to 10 kb in size. Nevertheless, the adenoviral *in vivo* delivery approach could also be employed in the context of a targeted nuclease approach in which the large cloning capacity of Ad vectors could be exploited to deliver both a nuclease and a homologous recombination template for a gene of interest using the same vector.

Another major advantage of the use of adenoviral gene transfer vectors is their ease of production. HDAd vectors can be easily produced to high titers and scale up is usually uncomplicated since the production of these vectors does not rely on large-scale transfection of genetic material. These advantages also result in a relatively low vector production cost, often orders of magnitude lower than those encountered for lentiviral or AAV vectors. A further advantage of an Ad vector platform is the ability to lyophilize vector preparations to ensure long term stability even when stored at 4°C⁶⁶.

Discussion

In vivo HSC gene transfer achieved using a minimally invasive manner and without the need for stem cell harvest or transplantation should simplify HSC gene therapy. Furthermore, by avoiding *ex vivo* HSC manipulation, the risk of cell differentiation or loss of homing/engraftment capability is avoided, as there is no need for the extraction and purification of target cells. *In vivo* gene delivery also bypasses the issue concerning the markers that enable the isolation of true primitive HSCs. *In vivo* gene transfer could target all HSCs, including those that are missed by different purification criteria. Finally, *in vivo* HSC transduction should eliminate the need for myeloconditioning with chemotherapy drugs. Preconditioning results in considerable early morbidity owing to transitory blood-cell depletion, immunodeficiency and mucosal damage, which place the recipient at risk of severe infection ⁶⁷. It also causes delayed morbidity owing to the risk of developing chemotherapy-induced secondary tumors and infertility. Several HSC gene-therapy trials have attempted to alleviate the morbidity associated with preconditioning by lowering the dosage and combination of chemotherapeutic drugs. However, the impact of changing these drug regimens on the risks and benefits of the therapy is yet to be determined in broader comparative studies and through long-term patient follow-up. Finally, the technical complexity and high cost of current *ex vivo* HSC gene therapy is a barrier to a widespread application for common diseases. As an example, Strimvelis™, a HSC gene therapy for ADA-SCID that has been approved for marketing in the European Union earlier this year, comes at a price of close to \$700,000 per treatment and the regimen is only available at a single center in Europe making access to it difficult even within the developed countries of Europe.

In vivo HSC gene transfer approaches do pose a number of potential problems, with the most important ones being due to direct contact between the gene transfer vector and the patient, which can trigger innate and adaptive immune responses. This problem is specifically pronounced for approaches involving intravenous vector injection. Viral vectors and non-viral nanoparticles are taken up by monocytes and macrophages in various tissues, specifically within the liver and spleen, and the incoming particle and DNA or RNA is sensed by innate immune mechanism. This results in release and activation of pro-inflammatory cytokines ⁶⁸. It is thought that AAV vectors provoke weaker innate immune responses than other viral vectors such as Ad or LV ⁶⁹, however, at this point no direct comparisons of these vector systems with regards to innate toxicity have been made following IV injection. In our studies with HDAd5/35++ vectors we found that the release of proinflammatory cytokines upon vector administration is increased by mobilization. Mobilization, specifically regimens that involve G-CSF, triggers acute leukocytosis so that more monocytes in the circulation can come into contact with the IV injected vector. Acute cytokine release however can be prevented by pre-treatment with glucocorticoids ⁷⁰, or anti-IL-6/anti-IL-6-receptor antibodies ⁷¹.

Another problem, specifically when using Ad and AAV vectors, is pre-existing B and T-cell responses against capsid proteins that can target infected cells or neutralize intravenously injected particles before they transduce HSCs and prevent re-administration. For example, the serum prevalence of neutralizing antibodies against AAV6 is 46% ⁷², and most people

have serum antibodies against Ad5 and specifically against the part of Ad5 that is present in Ad5/35⁷³. One alternative would be to use vectors derived from Ad35, as it is one of the rarest human serotypes with a seroprevalence of <7%⁷⁴. After intravenous injection of Ad35 vectors there is only minimal transduction (only detectable by PCR) of tissues, including the liver, in human CD46 transgenic mice⁷⁵ and non-human primates⁷⁶. Ad35 vectors have been used in clinical trials as vaccine vectors. Should pre-existing immunity remain a problem transient immunosuppression has been effective when countering humoral immunity in liver gene therapy trials that used AAV8 vectors³⁴.

Another potential drawback for intravenous HSC gene transfer is the requirement for higher doses of gene therapy vectors when compared to *ex vivo* approaches due to vector sequestration by the reticulo-endothelial system and the transduction of cells other than HSCs. Also, most mobilization treatments do not specifically mobilize HSCs into the blood stream. In our studies, we have shown transduction of lineage positive cells at day 3 after HDAd5/35++ injection⁶⁵. Even though these non-HSCs could express the transgene, their half-life, compared to HSCs, is short and the potential harm of them expressing the transgene would soon be lost. As a potential solution to this problem, vectors specifically targeted to HSCs could be generated. Some work has been done in this respect⁷⁷, whereby a lentiviral vector retargeted towards CD133 efficiently transduced cells with high multi-lineage reconstituting potential. Vector sequestration by macrophages of the liver, lung and spleen also remains a major unsolved obstacle to lowering IV injected vector doses, although the impact of the requirement of higher vector titers is highly dependent on the vector platform that is being used. For example, while Ad vectors are relatively cheap and easy to produce in large amounts, this will be more challenging for lentivirus and AAV vectors.

Another group of potential problems could be associated with the mobilization process that results in a significant increase in white blood cell counts. In our mouse studies we observed up to 20-fold higher WBCs one hour after G-CSF/AMD3100 mobilization. A significant fraction of mobilized cells might resettle in the spleen and this has, in rare cases, led to splenic ruptures in patients.

In summary, *in vivo* HSC gene therapy has the potential to move HSC directed gene therapy out of a few very specialized centers and may enable applications for common diseases at more affordable prices. However, before a widespread application of this approach can become a reality, more rigorous efficacy and safety studies are required.

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Key points

- Current protocols for hematopoietic stem gene therapy, involving the transplantation of ex vivo genetically modified HSC, are complex and not without risk for the patient.
- HSCs in the bone marrow are intricately connected with the bone marrow stroma, which creates a physical barrier to transduction with intravenously injected gene transfer vectors.
- Intraosseal injection of viral vectors has been shown to result in *in vivo* transduction of HSCs in mice. It might be more feasible and efficient in large animals and humans.
- A new approach that involves the mobilization of HSCs from the bone marrow, their transduction in the periphery, and return to the bone marrow, has shown first promising results.

Fig. 1. Schematic representation of four approaches for in vivo HSC gene therapy

The **upper left panel** shows an example of in vivo HSC gene editing using non-viral nanoparticles injected intravenously (IV) into mice. Site-specific triplex-forming peptide nucleic acids (PNA) interact site-specifically with genomic DNA via strand invasion resulting in the displacement of a DNA strand. This recruits endogenous DNA repair proteins to initiate site-specific modification of the genome when single-stranded donor DNAs are co-delivered. The **upper right panel** shows a study in dogs with a mutation causing SCID-X1. A foamy virus vector carrying the γ C gene capable of phenotypic correction of the disease, is delivered intravenously. The **lower left panel** depicts an intraosseal (IO) approach of gene delivery into bone marrow HSCs. In this example, a lentivirus vector carrying a human FVIII clotting factor expression cassette is injected via a syringe pump into the femurs of mice. The **lower right panel** shows an in vivo HSC gene transfer approach that involves the mobilization of HSCs from the bone marrow by subcutaneous (SC) injections of G-CSF/AMD3100. While HSC are in the peripheral blood, a capsid-modified, integrating helper-dependent adenovirus vector (HDAd5/35++) is injected intravenously. HSC transduced in the periphery home back to the bone marrow where they persist long-term.

Fig. 2. GFP marking in bone marrow HSCs after in vivo transduction

HSCs were mobilized in human CD46 transgenic mice by s.c. injections of human recombinant G-CSF (5 μ g/mouse/day, 4 days) followed by an s.c. injection of AMD3100 (5mg/kg) eighteen hours after the last G-CSF injection. Two integrating HD-AD5/35++ vectors at a total dose of 8×10^{10} viral particles were injected IV one hour after AMD3100. One Ad (HDAd-GFP) contains a GFP gene under the control of the EF1 α promoter, and the transgene cassette is flanked by inverted repeats that are recognized by the Sleeping Beauty transposase. The second Ad vector (HDAd-SB) expresses the hyperactive Sleeping Beauty transposase SB100x in trans. Animals were sacrificed 4, 8, 12, or 20 weeks after Ad transduction and bone marrow cells were isolated (n =10). **(A)** Shown is the percentage of GFP-positive HSCs (lin⁻, Sca1⁺, cKit⁺ -“LSK”) present in the bone marrow. **(B)** GFP expression in colony forming units. Bone marrow cells were lineage depleted via MACS followed by collection of GFP-positive cells via FACS. Cells were then plated in CFU assays and colonies were scored 12 days after plating.

Data from Richter M, Saydaminova K, Yumul R, et al. In vivo transduction of primitive mobilized hematopoietic stem cells after intravenous injection of integrating adenovirus vectors. *Blood* 2016; 128(18):2206–2217.