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Safe and Effective *In Vivo* Targeting and Gene Editing in Hematopoietic Stem Cells: Strategies for Accelerating Development

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On May 11, 2020, the National Institutes of Health (NIH) and the Bill & Melinda Gates Foundation (Gates Foundation) held an exploratory expert scientific roundtable to inform an NIH–Gates Foundation collaboration on the development of scalable, sustainable, and accessible HIV and sickle cell disease (SCD) therapies based on *in vivo* gene editing of hematopoietic stem cells (HSCs). A particular emphasis was on how such therapies could be developed for low-resource settings in sub-Saharan Africa. Paula Cannon, PhD, of the University of Southern California and Hans-Peter Kiem, MD, PhD, of the Fred Hutchinson Cancer Research Center served as roundtable cochairs. Welcoming remarks were provided by the leadership of NIH, National Heart, Lung, and Blood Institute, and Bill & Melinda Gates Foundation, who cited the importance of assessing the state of the science and charting a path toward finding safe, effective, and durable gene-based therapies for HIV and SCD. These remarks were followed by three sessions in which participants heard presentations on and discussed the therapeutic potential of modified HSCs, leveraging HSC biology and differentiation, and *in vivo* HSC targeting approaches. This roundtable serves as the beginning of an ongoing discussion among NIH, the Gates Foundation, research and patient communities, and the public at large. As this collaboration progresses, these communities will be engaged as we collectively navigate the complex scientific and ethical issues surrounding *in vivo* HSC targeting and editing. Summarized excerpts from each of the presentations are given hereunder, reflecting the individual views and perspectives of each presenter.

Keywords: gene therapy, gene editing, *in vivo* gene therapy, sickle cell disease, HIV

INTRODUCTION

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Welcoming remarks were provided by the leadership of NIH, National Heart, Lung, and Blood Institute (NHLBI), and Bill & Melinda Gates Foundation, who cited the importance of assessing the state of the science and charting a path toward finding safe, effective, and durable gene-based therapies for HIV and SCD. These remarks were followed by three sessions in which partic-

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This roundtable serves as the beginning of an ongoing discussion among NIH, the Gates Foundation, research and patient communities, and the public at large. As this collaboration progresses, these communities will be engaged as we collectively navigate the complex scientific and ethical issues surrounding *in vivo* HSC targeting and editing.

Summarized excerpts from each of the presentations are given hereunder, reflecting the individual views and perspectives of each presenter.

SESSION I: THERAPEUTIC POTENTIAL OF MODIFIED HSCs

In vivo targeting and gene modification of HSCs: lessons from *ex vivo* targeting of HSCs

Donald B. Kohn, MD

HSCs serve as a lifelong source for all blood cell lineages. They can be isolated from a patient, gene-corrected *ex vivo* by gene addition or gene editing, and reintroduced into the patient through autologous transplantation to treat inherited blood cell diseases such as SCD. In addition, *ex vivo* gene therapy has also been studied as a way to introduce specific anticancer reactivities, reduce myelosuppression by chemotherapy, and engineer resistance to HIV infection.

A critical functional goal of *ex vivo* gene therapy is to correct and engraft a high percentage of long-lived HSCs without introducing genotoxicity or losing their stem cell properties. Because of massive HSC proliferation once they begin to contribute to hematopoiesis, gene transfer must be permanent (or able to self-replicate rapidly as episomes to keep pace with cell division that has not been accomplished to date). This need for permanence has been addressed by stably adding complementary DNA, minigene, or genomic replacement genes or by gene editing an endogenous locus. Using transfection, electroporation, adenovirus, or adeno-associated virus (AAV) to introduce replacement genes has not worked very well, because of low efficiency, transience, and/or potential cytotoxicity. Thus, *ex vivo* gene therapy has come to rely on the integration of γ-retroviruses, lentiviruses, or transposons. Recent work with hybrid vector systems, such as Ad5/35 vectors to deliver transposons, may provide an additional approach.[4]

Over the past 20 to 30 years, several aspects of *ex vivo* gene therapy have evolved. Bone marrow harvesting has been mostly supplanted by the use of mobilized peripheral blood stem cells obtained by leukapheresis, where CD34 immunoselection is commonly used to enrich for HSCs and progenitor cells, and *ex vivo* culture conditions typically involve 1 to 4 days in serum-free medium with recombinant hematopoietic growth factors. Gene therapy has moved from γ-retroviral vectors, which require 5 days culture for transduction and mitosis for integration, to lentiviral vectors, which require only 1 to 2 days for transduction and can integrate even in nondividing cells.

Experience with *ex vivo* gene therapy offers several lessons that can be applied to *in vivo* targeting and modification of HSCs:

- Resting HSCs *in situ* may be resistant to viral or nanoparticle entry: The low-density lipoprotein receptor, which serves as the receptor for lentivirus vesicular stomatitis virus G protein (VSVG), is activated in CD34+ cells after 2 days in culture.[5] This receptor will not be available *in vivo*, on quiescent HSC *in situ*, so other targeting moieties will be needed.
- With *ex vivo* gene therapy, increased conditioning intensity increases engraftment of genetically modified HSCs. Whether efforts to ''make space'' are relevant to *in vivo* gene therapy is not yet clear. Results from thalassemia trials with *ex vivo* gene therapy have demonstrated that a higher percentage of gene-corrected cells can lead to improved disease response.[6]
- The presence of enhancers in the long terminal repeats of retroviral vectors has implications for safety. Concerns about high rates of genotoxicity from γ-retrovirus-mediated insertional oncogenesis led to the development of lentiviral vectors that are self-inactivating (SIN) because the enhancers have been removed. These vectors contain internal promoters to drive ubiquitous or cell lineage-specific expression of the transgene and have demonstrated an excellent safety profile in multiple trials to date.
- Gene disruption by nonhomologous end joining (NHEJ) in resting HSCs is more effective than homology-directed repair (HDR)-mediated gene editing. HDR is cell cycle specific and restricted to the S/G2 transition.[7] Thus, gene editing reagents would need to be delivered continuously over a month or so to achieve HDR in most of the cells as they stochastically enter cell cycle, or cells would need to be activated *in vivo* to become HDR competent.

Ex vivo genetic modification of HSC is complex and requires sophisticated facilities. In contrast, *in vivo* gene therapy holds the prospect of readily distributed therapies. The decades of experiences with *ex vivo* HSC gene therapy may inform new efforts for *in vivo*.

Lessons learned from *ex vivo* genetic therapies of hemoglobinopathies

Punam Malik, MD

Four trials have assessed lentiviral vector-mediated gene therapy targeting transfusion-dependent thalassemia

major, with varying degrees of success. All the vectors used were SIN. Therapy was assessed for the β^{87}/HVP569 vector, which encoded a mutant β^{T87Q}-globin gene driven by the β-globin promoter and the HS2, HS3, and HS4 enhancers, and flanked by two copies of the chicken HS4 core insulator elements. This vector was successful in only one of three patients after autologous transplantation after myeloablative busulfan conditioning.[8] Thereafter, an improved version of β^{87}/HVP569 that had no insulator and an improved titer, the LentiGlobin vector, showed success in thalassemia major patients with less severe thalassemia mutations (non-β0/β0 thalassemia) and three of nine patients with β^0/β^0 (or more severe β-globin mutations) reached transfusion independence.[6] Therapy with the GLOBE vector, which encoded the normal β-globin gene driven by the β-globin promoter and the HS2 and HS3 enhancers, resulted in reduced transfusion dependence in adults and transfusion independence in three of four children after intrabone marrow transplantation after myeloablative conditioning with treosulfan/thiotepa.[9] The results of the fourth trial, which assessed lentiviral therapy inserting the normal β-globin gene and the HS2, HS3, and HS4 enhancers after reduced intensity busulfan conditioning, have been presented at national meetings but are not yet published.

Ex vivo gene therapy trials in SCD have focused on the addition/reactivation of a potent antisickling globin by (1) the LentiGlobin vector encoding the β^{T87Q} mutant globin, after myeloablative busulfan conditioning cured one child who required chronic transfusions, but failed to show success with seven subsequent patients.[9,10] (2) Subsequent improvements in stem cell collection through plerixafor mobilization and apheresis, gene transfer efficiency, and vector production, along with monitoring of ablative dose exposure to busulfan, resulted in improved correction in subsequent patients. (3) The reactivation of γ-globin, which forms fetal hemoglobin or HbF by disrupting its repressor Bcl11a by a lentivirus vector encoding an shRNA to Bcl11a driven by the β-globin promoter and HS2,3 enhancers after myeloablative conditioning, has shown success in the initial patients—three patients with 9 to 18 months of follow-up showed a significant correction, with 24% to 30% HbF. (4) In another study, treatment of three patients with a lentivirus vector encoding a mutant γ^{G16D} (ARU-1801) driven by the β-promoter and HS2, 3, and 4 enhancer and reduced-intensity melphalan resulted in HbF expression levels of 8% to 20%, improving sickle cell symptoms substantially. (5) A study of *ex vivo* CRISPR-mediated editing of the Bcl11a erythroid-specific enhancer in sickle HSCs followed by a myeloablative busulfan conditioning and autologous transplant has shown robust success, with 46% HbF. The results of the last three trials have been presented at national meetings or are press releases but are not yet published.

The thalassemia and SCD trials offer several lessons applicable to *in vivo* gene therapy:

- High-quality high-titer vectors are important.
- Vector potency is important: high level of β/γ-globin gene expression in thalassemia and a potent antisickling globin gene in SCD.
- An adequate number of HSCs should be collected and mobilized.
- Efficient gene transfer (*i.e.*, vector copy number [VCN] of 1–3) or efficient editing into HSCs is important.
- Insertional oncogenesis has not been associated with the use of lentiviral vectors thus far. It is too early to assess off-target editing effects of gene editing trials.
- HSC maintenance during genetic manipulation will be a factor in the success of both *ex vivo* and *in vivo* gene therapy.

Both thalassemia and SCD gene therapy trials have also demonstrated the importance of a healthy bone marrow environment. The bone marrow niche contains mesenchymal stromal cells (MSCs) that provide physical support and regulate HSC homeostasis. Crippa *et al.* have shown that the bone marrow niche in patients with β-thalassemia contains high levels of iron and ferritin accumulation, and has a reduced primitive MSC pool because of increased reactive oxygen species production that results in a functional impairment in MSC supportive capacity.[11] These findings were consistent with a study by Aprile *et al.* that showed that mice with thalassemia had reduced HSC self-renewal capacity, reduced bone deposition, and reduced parathyroid hormone signaling, a key regulator of HSC activity, and that these defects could be rescued with *in vivo* activation of parathyroid signaling.[12] A study by Park *et al.* showed that the pathologic angiogenesis seen in mice with SCD could be reversed by a 6-week blood transfusion targeting sickle hemoglobin (HbS) to 30%.[13] Leonard *et al.* found that bone marrow from patients with SCD is characterized by inflammation, aggregation, and stress erythropoiesis, leading to suboptimal recovery of CD34+ cells.[14] A healthy bone marrow environment that supports engraftment may be particularly important if genetic manipulations damage the HSCs.

Toward *in vivo* targeting and gene modification of HSCs: regulatory science considerations

Peter Marks, MD, PhD

FDA regulates somatic and germline gene modifications that are used as therapeutics in humans, including both cell modification before administration and directly administered gene therapy vectors. Although somatic cell

gene editing is considered a reasonable approach for use at this time, an appropriations rider from Congress currently prohibits germline modifications in humans. When assessing gene modification therapies, FDA considers both scientific and regulatory factors. Scientific factors include efficiency of delivery, on- or off-target modification and expression, immune response to vector or transgene, insertional mutagenesis, and potential germline transmission. Regulatory factors include the nature and complexity of editing, safety issues, benefits versus risk, and monitoring after treatment.

FDA has developed seven guidance documents (including six in final form and one in draft form)[15] relevant to gene therapy. Three provide general guidance regarding the manufacturing or follow-up of gene therapy, one discusses long-term follow-up after administration of gene therapy, and three focus on specific clinical areas. The draft guidance, which might be particularly relevant to *in vivo* targeting and gene editing, focuses on interpreting the sameness of gene therapy products under orphan drug regulations. The orphan drug regulation guides incentives for the development of products designed to treat <200,000 individuals in the United States. Whether a gene therapy product receives an orphan drug designation and exclusivity depends on whether it is the same as existing products. Specifically:

- If two products have different vectors and different transgenes, they are considered different.
- If two products use the same vector but have different transgenes, they are considered different.
- If two products have the same transgene and promoter but two different vectors, they are considered different as long as the vectors are not very closely related.
- If two products use the same vector and same transgene but two different regulatory elements, the nature of the different regulatory elements determines whether the products are considered different.

Whereas personalized medicine involves finding the right drug to treat the patient, individualized medicine involves *creating* the right drug to treat the patient. Individualized medicine can be broken down further into customized products, which have the same mode of action for the same indication, and created products, which have different modes of action for different indications. A personalized pancreatic cancer vaccine that uses dendritic cells pulsed with an individualized peptide mixture is an example of a customized product; gene therapies using the same vector for two different lysosomal storage diseases would be created products. FDA considers gene editing may represent individualized therapy because it may address unique mutation. Addressing individualized gene therapy is an important area in light of the many rare hereditary and acquired diseases affecting <100 individuals per year globally.

The FDA's Center for Biologics Evaluation and Research (CBER) offers two programs to help sponsors, manufacturers, and researchers navigate the regulatory process. The INitial Targeted Engagement for Regulatory Advice on CBER producTs (INTERACT) program encourages early interaction with sponsors regarding preclinical, manufacturing, and clinical development plans and replaces the pre-pre–investigational new drug meeting. CBER Advanced Technology Team (CATT) meetings provide an interactive mechanism to discuss the advanced technologies or platforms needed to develop CBER-regulated biologics production. CATT meetings also allow sponsors access to early and ongoing interactions with CBER before regulatory submissions are filed.

Highlights from individual comments from participants

Vector copy number (VCN) is an important consideration for *ex vivo* gene therapy, which needs to balance efficacy and potency against safety and potential genotoxicity. Based on experience with *ex vivo* gene therapies for hemoglobinopathies, a VCN of 1 to 3 per cell, depending on the potency of the vector, is deemed to be best for lentiviral vector gene therapy. A higher number is not necessarily better and, in most cases, a VCN of 1 is sufficient to ameliorate disease.

To achieve a target VCN of 1 per cell, most *ex vivo* HSC transductions require a multiplicity of infection (MOI) of 3 to 10, with the MOIs calculated based on true titers, measured on the cell type in question. However, *in vivo* calculations will present further challenges, especially because $\sim$99% of vector is expected to become trapped in the liver. Moreover, *ex vivo* experience shows that VCNs tend to drop from what is initially observed *in vitro* to what is seen in patients after 6 to 12 months, making estimations based on expected long-term VCNs even more challenging.

The percentage of transduced cells is also a factor in the success of *ex vivo* lentiviral vector gene therapy. In hemoglobinopathy studies, a 20% to 30% correction among HSCs could alleviate symptoms or even cure disease. However, the threshold for gene editing likely depends on whether editing a single allele is sufficient. Moreover, although VCN in lentiviral gene therapy represents an average copy number per cell, gene editing to correct a mutation will have a maximum upper VCN of 2.

There are other important issues to consider with *in vivo* gene therapy. With *ex vivo* gene therapy, it is possible to be deliberate about which cells are exposed to vector. That will not be the case with *in vivo* therapy: cells might take a short time to be corrected, or they could become ''vector sinks.'' In addition, transduced cells could include both

stem cells and more differentiated progenitor cells, and corrected stem cells do not appear to have a selective advantage over progenitor cells. For example, endogenous erythropoietic progenitors are more editable and transfusable, so that patients may need to have endogenous erythropoiesis suppressed before vectors are introduced.

There is no specific guidance yet for *in vivo* gene editing; the regulatory requirements would likely be the same as they would be for other gene therapy products. FDA will consider whether genome editing occurs with systemic administration, how the gene product is targeted, and what the potential off-target effects are. Deciding what types of animal studies, if any, will be required presents a challenge; since the end result is the editing of human genes, studies in nonhuman primates (NHPs) with nonidentical genomes might not be helpful. Studies in human organoid models or humanized mouse models will likely provide more relevant information. Although the guidance Dr. Marks presented on interpreting the sameness of gene therapy products focused on viral vectors, interpretations would likely be similar for nonviral products.

SESSION II: LEVERAGING HSC BIOLOGY AND DIFFERENTIATION

Achieving selective and precise *in vivo* targeting and gene editing: key features of HSCs and their environment

Irving Weissman, MD

HSCs are the only cells in the hematopoietic hierarchy that self-renew and the only cells that provide lifelong engraftment of hematopoiesis.[16] After the embryonic–fetal boundary, they are carried forward only by self-renewal. They are rare, accounting for only 1 in 100,000 cells transplanted during HSC transplantations, and they are distinguishable by markers such as HoxB5 expression in mice and CD34 + 38–90+ in humans.[17,18]

In marrow, only ~5–10% of CD34+ are HSC. Moreover, CD34 selection does not eliminate T cells entirely and this has implications for allogeneic transplantation, as graft-versus-host disease (GvHD)-free transplantation requires pure HSCs without T cell contamination. About 95% of endogenous HSCs are in cell cycle stage G0. After classic cyclophosphamide/granulocyte colony-stimulating factor (G-CSF) mobilization, all marrow HSCs enter cell cycle and expand, and upregulate the expression of the macrophage ''don't eat me'' signal, CD47. They enter the bloodstream at G0/G1, home to the bone marrow through interactions between HSC integrin $\alpha_4\beta_1$ and sinusoidal endothelial vascular cell adhesion molecule, cross-fields of macrophages, and home to the CXCL12 niche through the HSC CXCR4 receptor.[19–24]

When HSCs are cultured *in vitro* with factors, they enter the cell cycle, entering G1 and at ~30 h entering S phase. HSCs in S/G2/M lose the ability to home to marrow when injected intravenously.[25,26] At 2–3 days of culture, the majority of the HSCs have become progenitor cells that cannot self-renew. Thus, only a small fraction of CD34+ cells to begin with are HSCs, and the HSCs within them that are in cycle home poorly.

The biology and environment of HSCs have implications for *in vivo* HSC gene modification. Retroviruses cannot transduce HSCs in G0/G1, but lentiviruses can.[27] Unlike cells, viral vectors cannot home to the bone marrow from blood, and it is unlikely that they can traverse marrow vessels to enter HSC niches. Current methods of mobilization will provide both vectors and G0/G1 HSCs in the blood only for brief intervals, and even mobilized HSCs represent a rare population. Although viral envelope proteins can promote fusion with HSC, allowing viral vectors to release their cargo into the cell, vectors for *in vivo* gene therapy will also need HSC-specific binding sites and off-target delivery, and integration could still be a problem. Nevertheless, producing lentiviral vectors with envelope ectodomains that bind HSC-enriched markers could enable *in vivo* gene transfer, and experimental manipulation of the mobilization regimen could prolong the residence time vector accessibility of HSC in the blood.

Enabling safe curative treatment of blood and immune diseases: lessons from the development of novel conditioning regimens for HSC transplantation

Agnieszka Czechowicz, MD, PhD

Although HSCs can facilitate the cure of many diseases that affect millions of people worldwide, they are vastly underutilized. It is thought that only ~10% of patients in the United States and Europe who could benefit from treatment with these powerful cells receive them today. This is most commonly done in the form of HSC transplantation, which is a highly complex process in particular limited by severe toxicities such as widespread tissue damage, life-threatening infections, and GvHD that can occur if HSCs are used from a donor. As part of this process, conditioning regimens are also needed to create ''space'' for HSC engraftment that are additionally genotoxic, leading to high rates of secondary malignancies. The ultimate goals for HSC-based therapies are 100% survival and cure rates, with easy resource-light treatments and no short- or long-term toxicities.

Existing conditioning regimens, which rely on chemotherapy and/or irradiation, are nonspecific and broadly ablative, leading to many of the current transplant-associated toxicities. Selective HSC-targeted conditioning regimens, in contrast, can be highly specific and nonmyeloablative, potentially dramatically improving outcomes for both allogeneic and newer autologous *ex vivo* HSC therapies. Dr. Czechowicz *et al.* have explored antibody conditioning approaches that use monoclonal antibodies targeting CD117/c-kit to eliminate endogenous HSCs by blocking critical signaling pathways,[28] directing

the immune system to kill those cells,[29] or poisoning them through antibody–drug conjugates (ADCs).[30] These antibody conditioning regimens have been shown to be able to result in a 99% depletion of endogenous HSCs, leading to up to 80% donor chimerism after transplantation in various mouse strains. None of the severe toxic effects seen with existing conditioning regimens have been observed with antibody-directed approaches. No significant cytopenias have been observed, immune memory post-conditioning has been shown to be preserved, and these approaches can be used across donors, even despite a partial or full major histocompatibility complex mismatch, when used in combination with transient immune suppression without causing GvHD.[31,32] Monoclonal antibodies targeting human CD117, combination CD117 and CD47, and an ADC targeting CD117 have all shown efficacy in NHPs and are now en route to the clinic.[33,34] ADCs targeting other HSC-specific antigens, such as CD90, CD110, and CD184, have also been developed and have shown efficacy in preclinical settings.[30] Although each of these antigens could be used for targeted depletion of endogenous HSCs, each could also potentially be used for *in vivo* HSC targeting.

These approaches may make HSC transplantation safer and easier, with a higher likelihood of efficacy without the current procedural risks. However, more development is needed, and more study is required to identify and understand short- and long-term consequences. Key lessons from this study may also be applicable to *in vivo* gene therapy, which may have various advantages compared with HSC transplantation, but it is important to recognize that disease specific therapies are likely to be needed, leading to additional challenges. Thus, research on improving HSC transplantation-based stem cell therapy as well as *in vivo* gene therapy continues to be needed.

Lessons from *ex vivo* targeting and gene modification of distinct HSC populations

Hans-Peter Kiem, MD, PhD

Dr. Kiem and colleagues have conducted autologous HSC transplantation studies in NHPs and have shown that HSC clones engraft early, within the first 2 months post-transplantation, and persist and contribute to hematopoiesis for years. Antibody characterizations in these studies have identified three distinct CD34+ cell populations, based on the presence or absence of CD45RA and CD90, markers that are conserved in humans, and further study has demonstrated that short- and long-term multilineage engraftment is driven exclusively by the CD34+CD90+ CD45RA− phenotype.[35] These findings are consistent with single-cell RNA analyses demonstrating that CD90+ cells are the most highly enriched CD34+ cell subpopulation that can be targeted for gene therapy or gene editing and represents $\sim$5% of the overall CD34+ cell population.[36]

Current gene editing approaches for SCD focus on reactivation of HbF, either by inactivating the HbF repressor Bcl11a or by recreating mutations associated with hereditary HbF persistence (HPFH). Dr. Kiem's group focused on the 13-nt HPFH deletion in the promoter region of the γ-globin gene that also contains the recently identified Bcl11a binding sites. Transplantation of CD34+ cells that have been edited by CRISPR/Cas9 to recreate this 13-nt HPFH deletion results in stable and persistent edited cells for up to 2 years after transplantation, and all observed deletions fall within the Bcl11a binding site. Robust engraftment of CRISPR/Cas9-edited HSCs was also observed when only CD90+ cells were edited and infused into NHPs that had undergone myeloablative conditioning, even though $\sim$10-fold fewer gene-edited cells were infused per animal than with cells enriched for CD34+. Strikingly, although the majority of infused cells were not edited, CD90+ cells that had been edited drove hematopoietic repopulation within 10 days.[37] These findings suggest that CD90 is an important marker for long-term repopulating HSCs and that a CD90-targeted vector or nanoparticle could be used for *in vivo* gene therapy or editing.

Highlights from individual comments from participants

Studies should explore whether cells that are mobilized and undergo targeted modification in the bloodstream can rehome sufficiently, in the absence of toxic conditioning regimens. Such studies should focus on rapid mobilizers that ideally leave a vacant stem cell niche and preserve enough of the biology to allow the cells to home to the right niche. *In vivo* mobilization approaches will likely benefit from an HSC selection strategy for corrected cells. For example, a vector could target an orphan receptor whose engagement transmits cell division signals to the stem cells, as well as using a targeting ligand with a high affinity to that orphan receptor, but not to other endogenous receptors.

A better understanding of the microenvironment and biology of HSCs will be needed to optimize mobilization and other approaches to facilitate *in vivo* editing of HSCs. CD47, CD90, integrin $\alpha_4\beta_1$, CXCR4, and other molecules have been established as HSC-specific cell surface markers. Studies in mice and in human bone marrow, blood, and fetal liver have shown differences in markers after HSC mobilization, but more studies are needed to compare the expression of HSC-specific markers in the bone marrow niche versus mobilized HSCs. In addition, all HSCs in mice enter the cell cycle and undergo upregulation of CD47, and daughter cells appear in the bloodstream, but it is not clear whether one daughter cell remains behind in the niche or whether both daughter cells are stem cells that enter the bloodstream. If the latter is true, then over time, all daughter cells could be available for transduction.

Studies in cord blood and fetal liver-derived stem cells in mice could help identify factors that play a role in such physiologic circulation of HSCs.

Using the right animal model is critical. For SCD, neonates are the ideal population to assess an intervention. Because the marrow physiology is protected by HbF expression at birth, this is an optimal time to ensure that mobilized edited cells will engraft. Humanized mouse models transplanted with fetal tissue would seem to be the most relevant model for studying the biology of human HSCs, blood-forming mechanisms, and potential interventions at this stage. However, although Dr. Irving Weissman and colleagues have been able to induce immune responses in such models, there are still questions about the quality of immune system development in these models. In addition, studies of disease-specific HSC biology are needed, not only in mouse models but also in larger animal models.

Because the HSC markers already noted are also present on other blood cells, ideally vectors could target multiple markers at a lower affinity, such that a high-affinity interaction between the vector and a cell requires avidity. An improved understanding of HSC biology, based on studies of purified true HSCs, will help to determine how specific vectors need to be. With nonviral approaches, saturating the bone marrow with specific vectors would likely transfect most cells in the niche, but it would also raise the likelihood of transfecting pure HSCs. However, vectors would need to be able to cross the endothelium, enter the bone marrow, and home to the right niche. Because these challenges will not exist with mobilized cells, researchers could identify a nontoxic way to mobilize HSCs, and then assess what fraction of cells have incorporated the vector and how long those cells remain in the blood.[4]

Although *in vivo* gene therapy is a bold and ambitious endeavor, research on other approaches also needs continued support and promotion. Purifying allogeneic stem cells *in vitro* from predefined human leukocyte antigen cohorts to produce an ''off-the-shelf'' reagent is one alternative. The reasons why fetal liver HSCs can enter the cell cycle without losing transplantation efficiency should also be explored. Fanconi anemia, in which there is a selection pressure so that only a small number of corrected cells are needed to alleviate disease, could provide a proof of principle to support studies on *in vivo* targeting and gene editing in SCD.

SESSION III: *IN VIVO* TARGETING OF HSCS

Nanoblades: a new tool for gene editing in hematopoietic cells

Els Verhoeyen, PhD

The CRISPR/Cas9 system is the preferred system for gene editing. One available tool for gene editing in HSCs, a lentiviral vector that expresses both Cas9 and the guide RNA, is not optimal, because of low titers, persistent Cas9 expression after transduction, and difficulties in achieving transduction.[38] Another available tool, electroporation with Cas9 plus guide RNA ribonucleic particles, works efficiently *in vitro*, but can be associated with a high level of toxicity and is not available as an *in vivo* application.[39,40]

Dr. Verhoeyen and colleagues have developed a nanoblade system based on HIV or murine leukemia virus to deliver editing reagents. This system makes a virus-like particle that is produced by cotransfection of constructs encoding the viral Gag-Pol proteins, a Gag protein fused to Cas9 and guide RNA expression cassettes.[41] These particles can be pseudotyped by viral glycoproteins, similar to the production of retroviral/lentiviral vectors. Pseudotypes are known to target HSCs: a pseudotype of lentivirus with baboon env-glycoprotein can target ASCT1 and ASCT2,[42] and a pseudotype of lentivirus with measles virus glycoprotein can target CD46.[43] Both pseudotypes have been associated with HSC transduction levels up to 100%. The measles lentivirus pseudotype can transduce HSCs efficiently without activation or without changing the population of mature cells, but application of this pseudotype *in vivo* will likely be difficult, because most people have been vaccinated against the measles virus.[44] In contrast, the baboon lentivirus pseudotype is a particularly attractive candidate, because it can transduce quiescent CD34+ cells and is resistant to human complement.[42]

In addition, incorporation of both the baboon and VSVG envelope proteins into nanoblades is associated with a higher concentration of Cas9, a higher rate of gene editing, and high reproducibility with respect to site-specific gene integration. This is a highly potent system, achieving with two guide RNAs simultaneously gene editing rates of 30% in IL-7–stimulated T cells, 50% in a suspension of K562 cells, 30% in HSCs, and up to 80% at locations targeting the MYD88 gene in HSCs, as assessed by PCR analysis.

Nanoblades can also be used to create primer knockins if double-stranded DNA or single-stranded DNA oligomers are placed at their surface.[41] In addition, a gene editing rate up to 60% and gene integration was observed when a nanoblade was combined with a recombinant AAV type 6 (rAAV6) donor carrying a green fluorescent protein (GFP) expression cassette with flanking homologous regions to the Wiskott Aldrich Syndrome gene locus. When the nanoblades and rAAV6 coding for the donor template were mixed with mildly prestimulated CD34+ cells and the medium changed after 8 h, the expression cassette was integrated at rates of 40% to 50% after 24 h, as assessed by stable GFP reporter gene expression after 3 and 12 days.[45]

Despite its many advantages, the baboon lentivirus pseudotype has some problems with specificity. Christian Buchholz, PhD, and colleagues have developed a lentiviral system pseudotyped with the Nipah virus glycoprotein

that can be retargeted through the insertion of targeting ligands, for example, to CD8+ cells.[46] Dr. Verhoeyen and Buchholz' teams have shown that this vector can modify CD8+ cells to redirect them to destroy B cells *in vivo*.[47] Combining nanoblades with this type of retargeting strategy for HSCs could prove especially potent.

In vivo transduction of HSCs in mobilized mice and NHPs using HDAd5/35++ vectors

André Lieber, MD, PhD

Gene delivery to HSCs *in vivo* involves the mobilization of HSCs from the bone marrow and introduction of an HSC-targeted vector while the cells remain in the periphery. This approach relies on intravenous or subcutaneous injections and avoids HSC collection, *ex vivo* cell culture, myeloablation or conditioning, and transplantation. It is also easier and portable. It should be noted, however, that without myeloablation, antitransgene immune responses can be a problem after *in vivo* transduction.

Dr. Lieber and colleagues have developed adenovirus derived HDAd5/35++ vectors, which can preferentially transduce HSCs in a humanized mouse model through their affinity for CD46 and can be produced at high yields with relatively low manufacturing costs.[48,49] Episomal HDAd5/35++ vectors incorporate CRISPR/Cas9 or base editors, and site-specific gene integration can be achieved by employing the *Sleeping Beauty* transposase or HDR. Initial experiments of the integrating HDAd5/35++ vectors focused on mobilization and transduction in mice and rhesus macaques, using standard mobilization with G-CSF and AMD3100. A large fraction of HSCs transduced in the periphery returned to the bone marrow but could not compete efficiently with nontransduced cells. This disadvantage led to a slow egress of transduced cells from the bone marrow. Thus, an *in vivo* selection step, based on an $mgmt^{P140K}$ mutant gene in the HDAd5/35++ vector, was added. This step led to transgene expression in 90% of peripheral blood mononuclear cells and a clear marking of all lineages in the bone marrow, with one to two integrated copies per cell and >1,000 different clones per mouse. HDAd5/35++ vectors expressing GFP, γ-globin, CRISPR/Cas9, and $mgmt^{P140K}$ are under evaluation in an ongoing NHP study supported by the Gates Foundation.[4,50,51] Pretreatment with dexamethasone and tocilizumab can block against cytokine storms, and no liver toxicity has been observed after intravenous introduction. So far, one animal had pre-existing acquired antibodies against a vector, resulting in low transduction. However, even at this low level of transduction, after *in vivo* selection, 90% of peripheral red blood cells were marked with γ-globin, although the level of γ-globin expression was low. Cells expressing high levels of GFP have been lost because of anti-GFP immune responses. An improved version of the vector is under evaluation in another animal, with the transduction level being 10 times higher than that seen in the first animal.

Evolving AAV vectors for stem cell applications

Aravind Asokan, PhD

AAVs contain a single-stranded genome packaged in a nonenvelope capsid. A wealth of available information on the biology and structural features of these viruses (*e.g.*, natural receptor usage and the cell surface–glycan interactions that dictate tropism) can be used to improve reagents for stem cell applications. The genome provides a template for HDR and can be designed to be self-complementary, as long as the appropriate hairpin loops and inverted terminal repeats are present on either side of the genome. Although these characteristics result in a narrow opportunity for integration in the presence of nucleases, the persistence of different forms can be leveraged for different applications. The surfaces of AAVs contain antigen footprints that have coevolved with cell surface receptor footprints. Although they allow AAVs to engage receptors on the cell surface, these coevolved footprints also tend to be targeted by neutralizing antibodies.[52] In addition, most AAVs distribute to the liver and other organs, so controlling expression in an AAV vector will require the targeting of more than one property.

Several reports suggest that AAV8 transduces mouse hepatocytes with robust efficiency. However, this serotype does not transduce human hepatocytes as efficiently in humanized mouse models. Dr. Asokan and colleagues have identified capsid surface features known to be used by other AAVs to engage cell surface receptors and have used this knowledge to generate a library of AAV variants, which the researchers screen over several rounds to identify strains that are able to amplify and replicate in mouse or human hepatocytes. This approach has allowed the team to expand the tropism of these AAV8 variants from mouse to human hepatocytes, creating a vector for liver gene transfer.[53] Using a similar approach, Dr. Asokan and colleagues have also generated libraries of AAV6 variants, across different donors and cell types, to overcome challenges associated with *ex vivo* transduction. Ultimately, the researchers aim to understand whether these novel variants will enable the delivery of cargo across a broad spectrum of applications, including *in vivo* gene editing.

Potential application of nanoparticles and other nonviral strategies for *in vivo* targeting of HSCs

Paula Hammond, PhD

Classic DNA plasmid delivery *in vivo* faces several barriers and challenges, including complexing DNA effectively to protect it from serum nucleases, ensuring systemic circulation while avoiding toxicity, directing the particle to bind the target cell of interest (which requires the particle to cross endothelial barriers), and routing to the nucleus. Several laboratories have attempted to use

delivery approaches similar to those used for small interfering RNAs. However, these approaches, although optimal for small RNAs, are associated with poor *in vivo* stability, poor transfection rates, and short half-lives in serum when used for larger nucleic acid structures.

In their study on cancer, Dr. Hammond and her colleagues have developed a layer-by-layer (LbL) nanoparticle incorporating a modular design, with a core containing chemotherapy, a layer containing a biologic to reduce the effectiveness of tumor defense mechanisms, an outer layer of hyaluronic acid for penetration into tumor cells, and a negatively charged polyelectrolyte stealth layer that is stripped off in the lower pH environment of the tumor. This system can be manipulated to change its interaction with cells and the environment. Early studies showed that the LbL nanoparticle enters tumor cells through receptor-mediated uptake but tracks to the endosome, leading (by processes that remain controversial)[54] to endosomal escape. The laboratory has, therefore, modified the nanoparticle so that it targets the tumor cells not only through the stealth layer but also through attached antibodies against specific cell types. A particle carrying an antibody against CD20 has shown enhanced uptake in lymphoma cells expressing CD20, leading to enhancements in treatment.

The Hammond laboratory is now applying the LbL approach to packaging large nucleic acids. The resulting nanoparticles contain a layer of negatively charged material, a core in which polyelectrolytes are used to condense large nucleic acids, and a clickable nondegradable synthetic polypeptide that can be tailored to enhance efficacy. Dr. Hammond and her colleagues have generated complexes containing plasmid DNA encoding CRISPR systems and have demonstrated effective transduction in tumor cells. The team is now collaborating with David Williams, MD, of Boston Children's Hospital, and David Scadden, MD, of Massachusetts General Hospital, to adapt this nanoparticle to target HSCs. A complex containing a GFP plasmid and an outer layer of anti-CD117/c-kit antibodies attached to hyaluronic acid has shown targeted uptake *in vitro* and in a mouse model with HSCs mobilized with G-CSF and AMD3100. Although most of the cells taking the nanoparticle were myeloid CD11b+ cells, a fraction of KSL cells in the bone marrow showed associations with the nanoparticle. A more significant fraction of cells showed uptake in peripheral blood, but only when they were mobilized. Thus, the c-kit nanoparticle is a promising strategy.

Targeting of lipid nanoparticles to deliver nucleoside-modified messenger RNA therapeutics

Drew Weissman, MD, PhD

The development of messenger RNA (mRNA)-based therapeutics has long been stalled because of mRNA's ability to activate intra- and extracellular immune sensors. To address this issue, Dr. Drew Weissman's laboratory has incorporated modified nucleosides into mRNA and purified it to remove contaminants remaining from *in vitro* transcription. Translation is increased about a 1000-fold with this nucleoside-modified mRNA, and when the mRNA is delivered to primary cells through lipid nanoparticles (LNPs), it transfects 80% to 90% of those cells, which produce protein regardless of whether they are dividing. The laboratory has developed targeting of the LNPs to specific cell types by cross-linking antibodies, receptor ligands, and fragments of antibodies to the end of the polyethylene glycol polymers attached to an incorporated lipid. Although this modification increases the size of the LNP by $\sim$20 nanometers, it does not affect the nanoparticle's surface charge, polydispersity, or function in delivering the modified mRNA.

Dr. Weissman and colleagues crosslinked anti-CD4 antibodies to an mRNA-LNP that would deliver a luciferase reporter protein to CD4+ T cells. After intravenous injection into mice, luciferase expression was observed in spleen and lymph nodes in T cells. The laboratory then measured gene-editing effector function by administering the anti-CD4 mRNA-LNP encoding Cre recombinase intravenously to Ai6 mice, which carry a genetic element encoding the ZsGreen reporter gene regulated by a STOP cassette flanked by loxP sites. Delivery of the LNP resulted in downregulation of CD4 on the T cells and robust ZsGreen expression in the spleen and lymph nodes. The laboratory has used different markers to target other T cell populations and found that a single LNP injection results in gene editing in 80% of the T cells in the spleen and lymph nodes. Scalable and facile systems are under development to facilitate pharmaceutical development.

Dr. Weissman's laboratory has also explored the ability of LNPs carrying nucleoside-modified mRNA to target the bone marrow. Initial studies linked R-spondin2, a secreted protein that activates the LGR4–6 receptors expressed in the bone marrow, to polyethyl glycol–lipid complexes in the LNP. Injection of the R-spondin2-linked LNP into mice resulted in increased translation by stem cells in the bone marrow. However, this targeting approach did not work in human studies. Dr. Weissman's laboratory is now collaborating with Dr. Kiem's group to use LNPs to deliver nucleoside-modified mRNA to CD90+ cells. Preliminary results demonstrate that CD90-targeted LNPs target $\sim$4% of CD34+ cells in bone marrow stem cell preparations, which is about equal to the percentage of CD90+ long-lived repopulating stem cells in stem cell preparations.

Considerations for *in vivo* delivery of gene editing reagents

Paula Cannon, PhD

Classic nuclease-mediated gene editing introduces a double-strand break in DNA that can be repaired by nonhomologous end joining to allow gene disruption, or

by HDR to provide greater options for precise gene editing. The CRISPR/Cas system requires delivery of both the Cas9 protein and a guide RNA, but a third component, a homology donor in the form of an oligonucleotide, plasmid, or DNA viral vector, is also required for HDR. Other editing tools that do not use nuclease-mediated double-stranded DNA breaks are also available. In dead Cas9 systems, mutations in Cas9 block its cutting activity while leaving its binding capabilities intact. Such modified proteins can then be linked with different effectors to regulate transcription or introduce epigenetic modifications into the targeted DNA. There are also other systems that precisely alter DNA sequences without requiring double-stranded DNA breaks: base editors combine a hemimodified Cas9 nickase with deaminases to create single base pair mutations and prime editing provides an all-in-one delivery solution for both the guide RNA and a homology template.

Packaging the components of these editing tools into vectors requires considerations of size limitations. For example, a prime editor comprises components up to 6.3 kb in size, whereas a meganuclease can be as small as 0.4 kb. Homology donor templates, when included, also increase size. This, in turn, affects the types of vectors that can be used to deliver the editing machinery since viral vectors have size constraints based on viral capsids, although nanoparticles are less constrained and can, therefore, accommodate more types of editing tools. In some cases, researchers may need to consider using vector combinations to deliver these different components.

Delivering gene-editing components *in vivo* also has temporal considerations, and the nature of the vector will influence the timing and duration of delivery. Because HSCs are primarily quiescent (G0) *in vivo*, relying on nuclease-mediated HDR, which is restricted to the G2/S phase of the cell cycle, may be challenging. Therefore, strategies such as NHEJ-mediated end capture, base editing, or prime editing may be more appropriate to consider for *in vivo* HSC targeting and gene editing.

Immune system barriers, making A versus making F, and an argument for *ex vivo* editing
Matthew Porteus, MD, PhD

Roundtable discussions throughout the day have noted the immune system as an important barrier to *in vivo* HSC gene editing. The innate immune system, which is both cell based and intracellular, responds to delivered editing constructs as if they were pathogenic; even modified mRNAs can induce type I interferon responses when introduced into CD34+ hematopoietic progenitor cells.[55,56] Thus, editing components must be targeted away from the *in vivo* immune system and packaged to avoid intracellular recognition. *In vivo* gene editing strategies also face challenges from the adaptive immune system. Approximately 30% to 40% of adults in Western countries have pre-existing immunity to AAV vectors, and ~60% to 70% of adult blood donors to the Stanford blood bank have pre-existing humoral and T cell immunity to *Streptococcus pyrogenes* and *Streptococcus aureus* Cas9.[57–60] Adaptive immunity can also be acquired: a single administration of AAV vector results in a massive humoral response that is cross-reactive with other AAV serotypes.[60,61] Mouse, dog, and NHP models have not been accurate predictors of human immune response to gene therapy protocols.

Because of the quiescent nature of HSCs, delivering high amounts of nuclease and donor DNA to induce HDR and convert the HbS allele to the HbA allele at a desired gene-editing rate of 20% is currently impossible. However, with strategies that deliver nucleases alone, it may be more possible to promote HbF production as achieving high *in vivo* transduction frequencies with high frequencies of *in vivo* HDR is not required.

Although studies in *in vivo* targeting and gene editing should be supported, *ex vivo* editing followed by transplantation should continue to be explored as a way to deliver therapies to patients around the world. Costs associated with patient identification, harvesting stem cells, and enriching for HSCs, gene correction, manufacturing and quality control of gene products, conditioning patients, and infusion and follow-up must be addressed. For example, good manufacturing practice (GMP)-qualified reagents are generally equivalent to research-grade reagents in effectiveness and safety, but the GMP qualification substantially (by 2 logs or more) increases the cost of the reagent. If manufacturing systems are converted to fully closed automated systems, core analytics are streamlined, and nontoxic conditioning regimens developed and implemented at lower costs, then the costs of reagents could be reduced to $3,000.

Highlights from individual comments from participants

Gene editing reagents need to be completely free of double-stranded RNA components, which can be generated during the manufacturing of Cas9-based systems and can interfere with editing efficacy. Although some laboratories have been able to manufacture reagents with almost no double-stranded RNA, many academic laboratories do not have good ways to measure these components and, therefore, cannot ensure their complete removal.

In the 1990s, Dr. Irving Weissman's laboratory showed that using hydroxyurea to synchronize endogenous HSCs resulted in cells that were close to S/G2/M when released. However, the laboratory did not test whether these cells could be mobilized for *in vivo* modification. In addition, hydroxyurea alters nucleoside composition and decreases HDR. Other approaches are, therefore, needed to put HSCs into cycle. A significant body of literature suggests that brief interferon signaling brings HSCs out of quiescence and that collapse does not occur unless infection is

prolonged. It is possible then that the interferon responses observed when reagents are added may be beneficial.

Manufacturing and scaling will become an issue for the clinical translation of viral delivery systems. With respect to nanoblades, it will be difficult to make a constitutive producer line based on VSVG, because it is cytotoxic, and inducible systems have not worked. So far, in collaboration with Axel Schambach (MHH, Hannover, Germany), Dr. Verhoeyen and colleagues have been able to produce stable envelope-packaging cell lines, including one that is not toxic, for BaEV. Similarly, HDAd5/35++ vectors will require a helper virus. Dr. Lieber and colleagues are working with a company to address these issues. Transduction efficiency with nonviral delivery should be explored further.

Cost will always be an important consideration. At present, *ex vivo* gene therapy requiring antibody conditioning is expensive. In the long term, however, changes in manufacturing reagents could reduce costs, while making lentiviral vectors or nanoparticles for *in vivo* delivery could be equally expensive. For many people with HIV and SCD in sub-Saharan Africa, even $3,000 would be beyond the allowable per capita expenditure. Though it may be possible to reduce costs even further, both *ex vivo* and *in vivo* targeting and gene editing approaches should be explored in parallel to provide the most practical solutions. Indeed, and as has been discussed in the context of target product profiles for an HIV ''cure,'' it may well be that there will be a sequential evolution of *ex vivo* and then *in vivo* approaches over time.[62]

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