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CRISPR/Cas9 genome editing in human hematopoietic stem cells

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Abstract

Genome editing via homologous recombination (HR) (gene targeting) in human hematopoietic stem cells (HSCs) has the power to reveal gene–function relationships and potentially transform curative hematological gene and cell therapies. However, there are no comprehensive and reproducible protocols for targeting HSCs for HR. Herein, we provide a detailed protocol for the production, enrichment, and *in vitro* and *in vivo* analyses of HR-targeted HSCs by combining CRISPR/Cas9 technology with the use of rAAV6 and flow cytometry. Using this protocol, researchers can introduce single-nucleotide changes into the genome or longer gene cassettes with the precision of genome editing. Along with our troubleshooting and optimization guidelines, researchers can use this protocol to streamline HSC genome editing at any locus of interest. The *in vitro* HSC-targeting protocol and analyses can be completed in 3 weeks, and the long-term *in vivo* HSC engraftment analyses in immunodeficient mice can be achieved in 16 weeks. This protocol enables manipulation of genes for investigation of gene functions during hematopoiesis, as well as for the correction of genetic mutations in HSC transplantation–based therapies for diseases such as sickle cell disease, β -thalassemia, and primary immunodeficiencies.

INTRODUCTION

Genome engineering is not only a powerful research tool, it is also being developed to cure human diseases, including those of the blood and immune system, most of which can be categorized as still having a great unmet medical need^{1–4}. *Ex vivo*–engineered nuclease-mediated gene editing by HR in hematopoietic stem and progenitor cells (HSPCs) can shed light on stem cell gene function through precise genetic manipulations, and can potentially define a curative strategy for currently incurable hematological diseases. The RNA-guided Type II CRISPR/Cas9 genome-editing system uses a single protein, Cas9, that is guided by a chimeric single-guide RNA (sgRNA) to target DNA through Watson–Crick base-pairing.

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AUTHOR CONTRIBUTIONS R.O.B. and D.P.D. contributed equally to this work, as well as designed and performed most of the experiments necessary for the methodological development presented here. M.H.P. directed the research and participated in the design and interpretation of the experiments and the writing of the protocol. R.O.B. and D.P.D. wrote the manuscript with help from M.H.P.

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

Because of its simplicity and robustness, it is becoming the most widely used engineered nuclease for editing of mammalian genomes^{5,6}. We have previously shown that the high editing performance of a plasmid-based CRISPR/Cas9 system in cell lines did not translate into high editing activity in primary cell types such as human primary T cells and HSPCs⁷. Protection of both sgRNA termini with chemically modified nucleotides increases sgRNA stability and renders the ‘all RNA’-based CRISPR/Cas9 system highly effective in primary HSPCs and T cells⁷. In addition, ribonucleoprotein (RNP) delivery of Cas9 precomplexed with chemically modified sgRNAs consistently increased activity in T cells^{7,8} and CD34⁺ HSPCs (R.O.B., D.P.D., and M.H.P., data not shown). Multiple publications have also shown efficient genome editing in T cells⁹ and HSPCs^{10,11} without modified sgRNAs in the context of Cas9 RNP delivery. In these studies, however, a direct comparison with synthetic sgRNAs with modifications was not performed, and it remains possible that the generally most active form of an sgRNA, even in the context of RNP delivery, is one that is synthetically manufactured with end modifications protecting against endogenous exonuclease degradation and innate immune stimulation.

Creating a locus-specific double-strand break (DSB) with engineered nucleases forms the foundation of genome editing^{12,13}. DSBs can be resolved by one of the two highly conserved competing repair mechanisms, nonhomologous end-joining (NHEJ) and HR¹⁴. NHEJ repair is the default pathway that functions throughout the cell cycle to repair breaks by ligation of DNA ends without end processing, sometimes resulting in small insertions or deletions (INDELs) at the site of the break. By contrast, HR is normally most active during the S or G2 phase of the cell cycle, when an undamaged sister chromatid is available to serve as an HR repair template. HR can be harnessed for creating precise DNA changes by supplying an exogenous DNA donor template, as long as the donor has homology arms that are identical to the region surrounding the DSB. This way, disease-causing single-nucleotide polymorphisms (SNPs) can be reverted or entire open reading frames can be inserted at specific genomic sites¹⁵ (Fig. 1). Although no studies using CRISPR and rAAV6 donors in CD34⁺ HSPCs have comprehensively investigated the impact of homology arm size and position relative to the nuclease cut site, studies using transcription activator–like effector nucleases (TALENs) and plasmid donors have found that maximal HR-mediated editing occurs when both the homology arms are at least 400 bp¹⁶.

Recombinant adeno-associated viruses (rAAVs) have been shown to naturally mediate high frequencies of HR in mammalian cells without stimulation of DSBs^{17–19}. Wild-type AAVs are non-enveloped single-stranded DNA viruses that consist of an ~4.7-kb genome encoding replication (rep) and capsid (cap) genes between 145-bp inverted terminal repeats (ITRs)¹⁹. Naturally occurring and engineered serotypes exist that have differential tropism, and serotype 6 has been shown to be the most efficient serotype for transduction of HSPCs and primary T cells^{20–22}. AAVs are dependent upon adenoviruses (helper viruses) to replicate *in cis*; however, packaging of rAAV vectors with user-defined DNA cargo is possible by cotransfection of the following three types of plasmids into a host cell line such as HEK293T: (i) transfer plasmids with the homology arms and transgene of interest between the two ITRs, (ii) rep/cap-encoding plasmids (in this protocol rep2/cap6), and (iii) helper plasmids encoding the adenoviral helper proteins (in this protocol Ad5). As rAAVs can generate a high vector copy number in the nucleus and avoid innate immunity, they are ideal

templates for HR following use of engineered nuclease-mediated DSBs in primary human cells. Accordingly, several recent reports have efficiently used rAAV6 as donor template for HR in primary human HSPCs^{21–24}.

Human hematopoiesis is the process that generates all blood and immune cells from HSCs with self-renewing capacity^{25,26}. Although progress has been made in identifying bona fide repopulating and self-renewing HSCs by immunophenotyping^{27,28}, the CD34⁺ cell surface marker has been readily used to identify a heterogeneous population of HSPCs. This population generally contains ~0.1–1% HSCs with long-term repopulation capacity (LT-HSCs). This capacity is normally experimentally tested in immunodeficient mice, e.g., in nonobese diabetic (NOD)-severe combined immunodeficiency (SCID)-gamma (NSG) mice, which lack innate and adaptive immunity to allow human cell engraftment in the mouse bone marrow²⁹. Several reports have recently shown that HR is more efficient in progenitor cells, as compared with LT-HSCs^{10,15,30,31}. Although current investigations attempt to augment HR rates in LT-HSCs, this is the biggest hurdle in accelerating clinical strategies for HR-based genome editing for blood and immune system disorders. We therefore devised a reporter-based enrichment paradigm that takes advantage of a log-fold-higher transgene expression after successful HR into the desired locus (compared with low AAV6 episomal expression), and this methodology could yield strikingly higher frequencies of modified cells in the transplanted mice (up to 97%)¹⁵. Thus, the enrichment methodology can solve the potential problem of inefficient HSC targeting.

In this protocol, we provide a reproducible methodology for achieving HR in HSPCs using CRISPR/Cas9 and rAAV6 homologous donor delivery. We describe in detail (i) sgRNA selection and AAV6 homologous donor design and construction, (ii) electroporation and transduction protocols, (iii) a flow cytometry-based strategy to enrich for a population of HPSCs with > 90% targeted integration, and (iv) *in vitro* and *in vivo* assays for determining HR frequencies in HSPCs. We also describe the use of the protocol in primary human T cells (Box 1). This is a comprehensive protocol for targeting human HSPCs for HR to investigate hematopoietic gene function and disease modeling, as well as preclinical development of HSC-based cell and gene therapies.

Box 1

Homologous recombination in primary human T cells ● TIMING 6–8 h hands-on, 5–7 d of culture

We have found that an identical protocol using the same reagents as described below can achieve up to 60% HR frequencies in T cells. Using CRISPR/Cas9 and AAV6, the transgene expression shift upon HR, which allows early enrichment of cells that have undergone HR (Fig. 2), is also apparent in T cells as early as Day 2 after electroporation and transduction.

The above figure shows representative FACS plots from Day 4 post electroporation of T cells with Cas9 RNP or without RNP (Mock) and then transduced with AAV6 vectors carrying an mCherry expression cassette flanked by homology arms for the targeted locus (Fig. 1a).

Reagents

- Ficoll-Paque PLUS (1.078 g/ml; GE Healthcare, cat. no. 17-1440-03)
- Pan T Cell Isolation Kit (Miltenyi Biotec, cat. no. 130-096-535)
- Anti-human CD3 antibody (BioLegend, cat. no. 317347)
- X-VIVO 15 with Gentamicin, L-Glutamine, and Phenol Red (Lonza, cat. no. 04-418Q)
- Human serum (Sigma-Aldrich, cat. no. H3667)
- Anti-human CD28 antibody (Tonbo Biosciences, cat. no. 70-0289-U100)
- IL-2, human (Preprotech, cat. no. 200-02)
- IL-7, human (BD, cat. no. 554608)
- Dynabeads Human T-Activator CD3/CD28 (Fisher Scientific, cat. no. 11132D)

Reagents

1. Purify PBMCs from buffy coats using standard Ficoll-based separation.
! CAUTION The use of tissue that is collected from human subjects requires approval by the local institutional review boards.
2. Isolate CD3⁺ T cells (Pan T cell isolation) from the PBMCs using the Pan T Cell Isolation Kit.
3. Directly after T cell isolation, stimulate cells by culturing them for 3 d at 1-million cells per well in a 24-well plate coated with antihuman CD3 antibody (plate precoated for 2 h at 37 °C with 300 µl of PBS with 10 µg of purified anti-human CD3 antibody per well) in X-VIVO 15 serum-free medium containing 5% (vol/vol) human serum, 1 µg/ml anti-human CD28 antibody, 100 IU/ml human IL-2, and 10 ng/ml human IL-7. Alternative to the CD3 and CD28 antibodies, human CD3/CD28 Dynabeads can be used at a bead-to-cell ratio of 1:1.

4. Three days after stimulation, electroporate cells and transduce with AAV6 donor vectors, as described in Steps 3–16 of the PROCEDURE using T-cell media as described above, but without anti-human CD28 antibody. As for CD34⁺ HSPCs, we strongly recommend that functional titration of the AAV vector be performed in HR experiments to identify the lowest MOI that yields maximum HR frequencies and high viabilities.

Comparison with other technologies

Just like Cas9, other engineered nucleases, such as zinc-finger nucleases (ZFNs), TALENs, and hybrid meganuclease-TALENs (megaTALs), can stimulate DSBs in mammalian genomes. However, Cas9 has two distinct advantages as compared with these other designer nucleases in stimulating DSBs in HSPCs. First, ZFNs, TALENs, and megaTALs are more cumbersome to construct and require an extensive molecular biology skill set. By contrast, the CRISPR/Cas9 system from *Streptococcus pyogenes* uses a simple 20-nt guide sequence to facilitate a locus-specific DSB. Furthermore, the chimeric sgRNA can be chemically synthesized with modifications at the ends that make it highly efficient in primary human HSPCs. Second, recombinant Cas9 protein can be easily produced and precomplexed with sgRNAs on the bench and delivered by electroporation as RNP complexes, which shortens nuclease exposure and provides a hit-and-run mechanism that decreases potential unwanted off-target DSBs.

Although rAAV6 can serve as a homologous donor template, there are other donor template platforms that have been shown to mediate efficient HR in HSPCs; these include single-stranded oligonucleotides (ssODNs)^{10,31} and integration-defective lentiviral (IDLVs) vectors³⁰. Although current literature may suggest that rAAV6 is a more efficient donor than IDLV²¹, a thorough comparison has yet to be performed. In comparison with ssODNs, which are usually ~50–200 bp in total size and can therefore mediate only small genomic changes, rAAV6 donor templates can mediate precise SNPs, as well as insert transgene cassettes up to ~4 kb in size if a single donor vector is used, or can insert even larger transgene cassettes if a sequential HR strategy with two donor vectors is used³². If single-point mutations are the desired genomic change, ssODNs may be a useful donor template for use in HSPCs, as they are easily produced and have been shown to work well *in vitro*¹⁰. However, ssODNs are too small to encompass an expression cassette for a reporter gene, and are therefore not compatible with the enrichment protocol for HSCs with precise targeted integration by flow cytometry that we outline in this protocol, which we believe is the key step for eliminating the greater number of nontargeted HSCs that outcompete targeted HSCs for engraftment in the host bone marrow.

Precise genome editing in HSCs has several advantages compared with conventional lentiviral vector (LV)-based gene transfer methods. First, genome editing maintains endogenous regulatory elements, preserving physiologic spatiotemporal regulation of gene expression^{1,3}. Second, as LVs integrate semirandomly within the genome, the possibility always remains of insertional mutagenesis near or in oncogenes and/or tumor suppressor genes, confounding experimental results and possibly, although fortunately not yet described, leading to leukemogenesis in therapeutic settings. Third, semirandom lentiviral

integration leads to expression heterogeneity among cells, which can be a major confounder in understanding gene-cell function in a heterogeneous population such as HSPCs. For gene therapy, this creates a population of cells with differential potency, potentially requiring higher cell doses or higher vector copy numbers. Fourth, LV-based gene addition does not allow targeted gene knockout by integration of a reporter expression cassette into the gene of interest, which enables tracking and enrichment of knockout cells. These reasons make genome editing the preferred genetic manipulation strategy to elucidate HSC gene function, as well as to correct disease-causing genetic mutations for HSC-based therapies.

Limitations of the protocol

One of the main limitations of the CRISPR/Cas9 system is the need for a protospacer adjacent motif (PAM) sequence within the gene of interest; however, the 5'-NGG-3' for SpCas9 can on average be found every 8–12 bp in the human genome and thus does not usually hinder application³³. Cas9 variants have been engineered with other PAM specificities that might circumvent this problem, although these engineered variants have not as yet been shown to mediate high levels of editing in primary human cells^{34,35}. In addition, appropriate donor design (**Supplementary Methods**), as described in this protocol, can also usually solve the problem when the CRISPR/Cas9 nuclease site is at a distance from the desired change. A limitation of our enrichment methodology is the need for an exogenous promoter driving the reporter transgene for enrichment (such as GFP or truncated nerve growth factor receptor (tNGFR)) for purifying targeted HSPCs early in the manufacturing process via flow sorting. We do note that the enhanced transgene expression after HR seems to be independent of the chosen promoter, which would allow the use of promoters with varying strengths in HSPCs or inducible promoters. Furthermore, it is possible that active endogenous promoters can be used to drive expression from enrichment cassettes for purifying targeted HSPCs. Nevertheless, the enrichment scheme is fundamental in removing nontargeted HSCs that can outcompete targeted HSCs for repopulation in the host bone marrow.

Experimental design

sgRNA design—Excellent protocols for designing and testing sgRNAs have been described previously³³. Here, we outline the key steps for identifying and characterizing a highly active locus-specific sgRNA, which is key to achieving optimal HR frequencies in HSPCs (**Supplementary Methods**). In brief, we routinely screen four to eight sgRNAs (depending on the available PAM sites in the targeted region) in the immortalized K562 cell line. Once a potent candidate sgRNA has been identified, a chemically modified synthetic sgRNA is ordered (Reagents section) from TriLink Biotechnologies (1-μmole minimal synthesis) or Synthego (3-nmole minimal synthesis; as new commercial sources for full-length synthetic modified sgRNAs become available, these might be used as well) and is functionally validated in human CD34⁺ HSPCs. Alternatively, we routinely bypass the functional screen in K562 cells and directly screen a panel of chemically modified synthetic sgRNAs in CD34⁺ HSPCs. Once high activity (as measured by the frequency of insertions and deletions using tracking of INDELs by decomposition (TIDE) analysis) of an sgRNA in HSPCs has been confirmed, a homologous donor template to introduce a desired genomic change is designed and cloned into an AAV vector plasmid. In general, the CRISPR cut site

(between base pairs 17 and 18 of the 20-nt complementary sequence of the sgRNA) should ideally be located as close to the intended genomic change as possible³⁶, although with proper design of the donor vector, we have observed substantial activity at a distance of 40 bp from the cut site¹⁵. In our experience, the farther the homology arms from the break site, the lower the HR efficiencies. When adding transgene expression cassettes into a safe harbor locus, the location of the sgRNA is less restricted, but if adding full cDNA cassettes to be driven by the correct endogenous regulatory context, e.g., directly into the start codon of a gene, the sgRNA target site should be located as close to the intended integration site as possible.

Donor design—The standard HR donor design is two homology arms (symmetric in length) that flank the transgene expression cassette or the mutations that will be introduced (Fig. 1a; **Supplementary Methods**). Homologous DNA donor design has been well described in excellent protocols referenced here^{37,38}. Here, we will describe, in brief, the key concepts to consider when designing an AAV6 homologous DNA donor for CRISPR/Cas9-stimulated HR in HSPCs. Furthermore, details outlining the cloning of HR donors into an AAV plasmid backbone, and the production and purification of AAV vector particles are presented in the **Supplementary Methods**. The AAV packaging capacity of 4.7 kb limits the size of the donor. The ITRs and homology arms are indispensable elements of the vector, and the ITRs are combined at ~270 bp. We recommend homology arms no < 400 bp each, and we have not observed any substantial advantage of extending the length¹⁶, nor any advantage to using self-complementary AAV6 (double-stranded) over single-stranded AAV6 templates (R.O.B., D.P.D., and M.H.P., data not shown). This leaves ~3.6 kb for the insert, so for HR of large transgenes or multicistronic cassettes, careful consideration must be made when designing this. For small genome modifications, such as introduction of SNPs, we recommend 1,200-bp homology arms to keep the total vector size > 2.4 kb, which is greater than half of the AAV packaging capacity and prevents concatemer packaging³⁹ (Fig. 1b). If the HR process is to integrate DNA exactly at the double-strand break, the arms should be split at the CRISPR cut site (Fig. 1a). If the desired modification is separate from the CRISPR cut site, the homology arms should flank the region between the cut site and the site of the modification (Fig. 1b,c). Two important considerations must be made in this situation: (i) If the target site is not disrupted by the introduced modification, Cas9 might introduce an INDEL after HR has taken place. To avoid this, mutations must be introduced (synonymous, if necessary) in the PAM or the sgRNA target site of the donors⁴⁰ (Fig. 1b). (ii) If there is sequence homology between the donor and the target DNA in the region between the cut site and the introduced changes, termination of the HR process may occur due to the nascent DNA strand leaving the donor and annealing back to the chromosomal DNA in this region. To avoid this, mutations (synonymous, if necessary) should be introduced to minimize homology in the region between the cut site and the site of the desired change (Fig. 1b). Several studies have shown that mutations at codon wobble positions suffice to prevent premature HR termination^{15,41}. If a cDNA cassette is to be integrated at the exact position after the start codon, the sgRNA target site should be located as close to the start codon as possible to minimize the gap between the start codon and the cut site, which, if too large, can negatively influence HR rates (Fig. 1c).

rAAV vector production—Excellent and comprehensive protocols have already described the production, purification, and titering of recombinant AAVs (rAAVs)^{38,42}, and these provide sufficient resources to make rAAV6 (**Supplementary Methods**). We have detailed the critical steps for rAAV6 vector production and purification in the **Supplementary Methods**. In brief, rAAV vectors are commonly produced in HEK293T cells by the triple-transfection method, which involves transfection of (i) an ITR-containing transfer plasmid, (ii) a plasmid expressing the adenoviral proteins and RNAs required for helper functions, and (iii) a plasmid expressing the AAV rep and cap proteins that define the serotype. We use a transfer plasmid carrying ITRs from AAV2 (Reagents) and assemble the donor plasmid by standard Gibson assembly of the linear transfer plasmid backbone with terminal ITRs and PCR fragments containing the homology arms amplified from genomic DNA and the insert amplified from a desired expression plasmid. SNP donors can be made in a similar manner using standard site-directed mutagenesis techniques to introduce the SNPs. For AAV production, we use the dual-plasmid transfection system previously described³⁸, in which the required adenoviral and AAV genes are combined on a single helper plasmid, pDGM6, which was described previously⁴³ and can be obtained from the Russell lab at the University of Washington (Reagents). We routinely use rAAV vectors purified from iodixanol gradients for HR experiments in HSPCs, but note that other purification methods work as long as they produce pure rAAV preparations with a low proportion of empty capsids. A high-titer preparation is essential to achieving high transduction rates and avoiding toxicity induced by the dilution of the stem cell culture media when using high volumes of AAV vector. We recommend that the total volume of AAV not exceed 20% of the total culture volume (ideally, < 10%). For titering the AAV preparations, we suggest using quantitative PCR on the ITRs to quantify the number of vector genomes as described previously⁴⁴. It is important to note that the titer obtained is not a functional titer. A wide range of different multiplicities of infection (MOIs, i.e., vector genomes (vg) per cell) have been used in studies performing HR in HSPCs using AAV6. For example, Wang *et al.* used MOIs of $1\text{--}10 \times 10^3$ vg/cell²¹, whereas De Ravin *et al.* used MOIs of $1\text{--}3 \times 10^6$ vg/cell²³. We believe that this large difference is due to differences in titering methodology and/or differences in AAV purity. We suggest that AAV titers be normalized to the AAV2 Reference Standard Material obtainable from the American-Type Culture Collection (ATCC, cat. no. VR-1616)⁴⁵. This will allow more-streamlined titers, but will not address differences in AAV purity. We recommend that functional AAV titration be performed in HR experiments in HSPCs to identify the lowest MOI that yields maximum HR frequencies with minimal cellular toxicity.

Enriching cells that have undergone homologous recombination (Steps 47–52)—An intrinsic feature of single-stranded AAV is that expression relies on second-strand synthesis, which subsequently produces dsDNA that is transcriptionally competent. We have recently reported on the observation that episomal AAV6 expression leads to low levels of reporter gene expression, even when driven from constitutive strong viral promoters in primary cells¹⁵. By contrast, we found that the reporter expression was increased more than a log-fold after HR-mediated chromosomal integration of the same expression cassette (Fig. 2; **Supplementary Fig. 1**). We have found that this phenomenon does not depend on cell type, target locus, exogenous promoter, or designer nuclease system used. The reporter shift

is apparent as early as 24 h after electroporation and transduction (peaks at 3–4 d), and depends on transgene expression kinetics and proliferation status of the cells. We have used this shift in transgene expression to sort cells with reporter^{high} expression and have obtained a purified targeted population with up to 99% purity. Importantly, we have shown in serial transplants that this enriched targeted population contains LT-HSCs.

Colony-forming unit assay and clonal genotyping (Steps 22–46)—The colony-forming unit (CFU) assay is a progenitor assay that assesses the potential of progenitor cells to form colonies in semi-solid methylcellulose media. This assay monitors the number of progenitor cells in the targeted population, as well as the proportion of lineage-committed progenitors (myeloid: CFU-GM; erythroid: BFU-E and CFU-E) and multilineage progenitors (mixed myeloid and erythroid: CFU-GEMM). It also enables the analyses of clonal genotypes by PCR screening of colonies for targeted integration of a transgene expression cassette. For this, an ‘In–Out’ PCR approach is used, in which one primer is located in the targeted genomic locus outside the region of the homology arm and the other primer is located inside the transgene cassette (Fig. 3a). Preferably, two PCRs are performed, identifying both the 5′ and the 3′ junction of the integration. If desired, a third primer can be included for identifying alleles without integration. This primer should be located in the genomic region on the opposite side of the sgRNA target site from the primer outside the homology arm (Fig. 3). It is critical that this primer be located at a distance of at least 50 bp away from the sgRNA cut site, as INDELs may otherwise disrupt the primer-binding site. HR frequencies for integration of SNPs can be assessed by droplet digital PCR or sequencing approaches (such as next-generation sequencing or TOPO cloning), or, if the genomic modifications generate a novel restriction site, restriction fragment length polymorphism (RFLP) analysis can quantify HR rates as described thoroughly before³³. The same primer design as outlined above should be used with one primer located outside the region of the homology arms.

Repopulation assay in transplanted immunocompromised mice (Steps 53–65)—Importantly, the CFU assay does not analyze the presence of HSCs with self-renewal and multilineage capacity, and for such verification, transplantation into immunocompromised mice is necessary. Although several mouse strains have been used for human hematopoietic repopulation, we recommend using the NSG strain, which is highly supportive of human engraftment and hematopoietic repopulation. If using other strains, more cells may need to be transplanted for robust engraftment. If transplanting bulk, nonenriched cells (RNP electroporation + AAV6 transduction) or RNP-electroporated CD34⁺ HSPCs without AAV6 donor transduction, transplantation can be performed as early as 2 h to 2 d after electroporation. HSPCs enriched for targeting via a reporter gene are transplanted directly after enrichment, when the frequency of reporter-positive cells peaks (Steps 17–21). Different standards for confirming long-term repopulating capacity of stem cells in NSG mice have been reported from 12–20 weeks in both primary and secondary recipients, respectively. Our recommendation is to assess engraftment 16 weeks after primary transplantation, and optionally perform secondary transplants and analyze these after 12 weeks to confirm true repopulating capacity.

Controls—As a positive control for Cas9/sgRNA activity in CD34⁺ HSPCs, we refer to the published sgRNA targeting the *HBB* gene and the matched *HBB* donor vector encoding GFP¹⁵. For all targeting experiments, a mock-electroporation control (no Cas9 RNP) is essential. This should be split into two wells: one that receives the AAV donor and one that does not. The latter is used for flow cytometric gating of reporter⁺ cells, and the former is used to set the reporter^{high} gate (Fig. 2). Similarly, for the colony-forming unit assay and engraftment studies in NSG mice, a mock control will serve as a positive control and a potency reference for colony formation/distribution and engraftment.

MATERIALS

REAGENTS

HSPC cell culture

- SFEM II (StemCell Technologies, cat. no. 9655)
- IL6 (PeproTech, cat. no. 200-06)
- SR1 (CellagenTech, cat. no. C7710)
- UM171 (StemCell Technologies, cat. no. 72914)
- Flt3L (PeproTech, cat. no. 300-19)
- TPO (PeproTech, cat. no. 300-18)
- SCF (PeproTech, cat. no. 300-07)
- FBS (Fisher Scientific, cat. no. 10438026)
- Penicillin–streptomycin (Fisher Scientific, cat. no. 15070063)
- Fresh or frozen CD34⁺ human HSPCs (either acquired per institutional protocol or purchased from various commercial sources, including All Cells (CB-derived, cat. no. CB008F; mPB-derived, cat. no. mPB015F)). If available, investigators can purify CD34⁺ HSPCs, for example, from umbilical cord blood, bone marrow aspirates, or mobilized peripheral blood per institutionally approved protocols and following the FACS-based or immunomagnetic microbead technology protocols presented here⁴⁶

! CAUTION The use of tissue that is collected from human subjects requires approval by the local institutional review board.

▲ CRITICAL CD34⁺ HSPCs from different sources are available from various commercial vendors either as fresh or frozen samples. Frozen cells should be thawed according to the vendor's specifications.

Genome-editing reagents

- Cas9 recombinant protein with nuclear localization signal (NLS; Life Technologies, cat. no. B25641 or IDT, cat. no. 1074182)

- Synthetic sgRNA, identified and designed as described in the **Supplementary Methods**, with three terminal nucleotides modified with 2'-O-methyl phosphorothioate (bold in the sequence below). The 20-nt targeting region is indicated by Ns (TriLink, custom RNA synthesis, quote by request, or Synthego, synthetic sgRNA; example below).

NNNNNNNNNNNNNNNNNNNNNNNGUUUAGAGCUAGAAAUAGCAAGUU
AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUC
GGUGCUUUU **▲ CRITICAL** Although synthetic sgRNAs without modified nucleotides are fully functional when complexed with RNPs, in our experience, they are less efficient than chemically modified sgRNAs.

- K562 cell line (ATCC, cat. no. CCL-243) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.
- ATP-disodium salt (Sigma-Aldrich, cat. no. A3377)
- $MgCl_2 \cdot 6H_2O$ (Sigma-Aldrich, cat. no. M2393)
- Nuclease-free ultrapure water
- KH_2PO_4 (Sigma-Aldrich, cat. no. P5655)
- $NaHCO_3$ (Sigma-Aldrich, cat. no. S5761)
- Glucose (Sigma-Aldrich, cat. no. G8270)
- RPMI 1640 (GE, cat. no. SH30027.01)
- Human T Cell Nucleofection Kit (Lonza, cat. no. VPA-1002)
- P3 Primary Nucleofection Kit (Lonza, cat. no. PBP3-00675) **▲ CRITICAL** Note that cuvettes or strips can be reused at least ten times if rinsed thoroughly with ultrapure water and sterilized with ethanol or exposure to UV light for 15 min.
- KCl (Moltox, cat. no. 26-645)
- $MgCl_2$ (Sigma-Aldrich, cat. no. M2393)
- Na_2HPO_4/NaH_2PO_4 , pH 7.2 (Boston BioProducts, cat. no. BB-180)
- Mannitol (Sigma-Aldrich, cat. no. M4125)

Analysis of HR efficiencies

- MethoCult Enriched (StemCell Technologies, cat. no. 04435)
- Zero-Blunt TOPO Cloning Kit (Fisher Scientific, cat. no. K280020)
- Propidium iodide at 1 mg/ml (Sigma-Aldrich, cat. no. P4864)
- Immunodeficient NOD-SCID Il2rg^{-/-} (NSG) mice, preferably female to support the highest engraftment⁴⁷, 6–8-weeks old (The Jackson Laboratory) **▲ CRITICAL** To achieve optimal engraftment rates, we highly recommend using

the NSG strain with *IL2RG* knockout, which supports higher engraftment as compared with the NOD-SCID strain ! **CAUTION** The use of animals for research purposes requires approval from the relevant national and institutional regulatory bodies.

- PBS, 1×, without calcium and magnesium (VWR, cat. no. 45000-448)
- QuickExtract DNA Extraction Solution (Epicentre, cat. no. QE09050)
- GeneJET Gel Extraction Kit (Fisher Scientific, cat. no. K0691)
- Agarose (Fisher Scientific, cat. no. 17852)
- 50× TAE buffer (Bio Rad, cat. no. 1610773)
- 1 kb Plus DNA ladder (Fisher Scientific, cat. no. 10787018)
- 100-bp DNA ladder (Fisher Scientific, cat. no. SM0323)
- Phusion Green Hot Start II High-Fidelity PCR Master Mix (Fisher Scientific, cat. no. F566S)
- Midori Green Nucleic Acid Staining Solution (Bulldog Bio, cat. no. MG04)
- Red blood cell lysis buffer, 1× (eBioscience, cat. no. 00-4333-57)
- Dextran (Sigma-Aldrich, cat. no. 31392)
- DNase (deoxyribonuclease I; Worthington Biochemical, cat. no. LS002007)
- Heparin sodium (Fisher Scientific, cat. no. BP2425)
- Ficoll-Paque Plus (GE, cat. no. 17-1440-03)
- Trypan Blue (Corning, cat. no. 25-900-CL)

Antibodies (for immunophenotyping HSPCs or HSCs *in vitro*; GFP compatible)

- Anti-human CD34-APC or CD34-APC-Cy7 (if using tNGFR as reporter), Clone 561 (BioLegend, cat. no. 343608 or 343614) ▲ **CRITICAL** If using tNGFR as reporter, use anti-human CD271 (tNGFR)-APC Clone ME20.4 (BioLegend, cat. no. 345108)

Antibodies (optional for staining for HSC phenotypic markers)

- Anti-human CD45RA-BV605 Clone HI100 (BioLegend, cat. no. 304134)
- Anti-human CD90-BV421 Clone 5E10 (BD, cat. no. 562556) ▲ **CRITICAL** Optionally, users can switch to CD90-FITC Clone 5E10 (BD, cat. no. 561969) if sorting different CD34⁺ subpopulations before targeting (**Supplementary Fig. 2**).
- Anti-human CD38-PE-Cy7 Clone HIT2 (BD, cat. no. 560677)
- Anti-human CD-123-PE (BD, cat. no. 554529)

Lineage antibodies (optional; for staining for HSC phenotypic markers)

- Anti-human CD2-PE-Cy5 (BD, cat. no. 555328)
- Anti-human CD3-PE-Cy5 (BD, cat. no. 555341)
- Anti-human CD4-PE-Cy5 (BD, cat. no. 555348)
- Anti-human CD8-PE-Cy5 (BD, cat. no. 555368)
- Anti-human CD16-PE-Cy5 (BD, cat. no. 555408)
- Anti-human CD19-PE-Cy5 (BD, cat. no. 555414)
- Anti-human CD20-PE-Cy5 (BD, cat. no. 555624)
- Anti-human CD56-PE-Cy5 (BD, cat. no. 555517)
- Anti-human CD235a-PE-Cy5 (BD, cat. no. 559944)
- Anti-human CD14-PerCP (BD, cat. no. 340585)

Antibodies (for human engraftment analysis in transplanted mice; GFP compatible)

- Human TruStain FcX (BioLegend, cat. no. 422301)
- Anti-human HLA-A/B/C-APC-Cy7 Clone W6/32 (BioLegend, cat. no. 311426)
- Anti-human CD45-V450 Clone HI30 (BD, cat. no. 560368)
- Anti-human CD33-PE Clone WM53 (BD, cat. no. 555450)
- Anti-human CD19-APC Clone HIB19 (BD, cat. no. 555415)
- Anti-mouse Ter119 PE-Cy5 Clone TER-119 (eBioscience, cat. no. 15-5921-82)
- Anti-mouse CD45.1-PE-Cy7 Clone A20 (eBioScience, cat. no. 25-0453-82) ▲ **CRITICAL** If using tNGFR as reporter, use anti-human CD271 (tNGFR)-APC Clone ME20.4 (BioLegend, cat. no. 345108) and switch anti-human CD19-APC antibody to anti-human CD19-FITC (clone HIB19, BD, cat. no. 560994).

Antibodies (for immunophenotyping HSCs *in vivo*; GFP compatible)

- Anti-human CD45-V450 Clone HI30 (BD, cat. no. 560368)
- Anti-human CD10-APC-Cy7 Clone HI10a (BioLegend, cat. no. 312212)
- Anti-human CD38-PE-Cy7 Clone HIT2 (BD, cat. no. 560677)
- Anti-human CD34-APC Clone 8G12 (BD, cat. no. 340441)
- Anti-mouse CD45.1-PE-Cy5 Clone A20 (eBioScience, cat. no. 15-0453-82)
- Anti-human CD271 (tNGFR)-APC Clone ME20.4 (BioLegend, cat. no. 345108) ▲ **CRITICAL** Use this if using tNGFR as reporter.
- Anti-human CD34-FITC Clone 8G12 (BD, cat. no. 348053) ▲ **CRITICAL** Switch anti-human CD34-APC antibody to this if using tNGFR as reporter.

EQUIPMENT

- Steriflip 0.45- μ M filter (Millipore, cat. no. SE1M003M00)
- Electroporation cuvettes (if using Amaxa Nucleofector IIb; VWR, cat. no. 89047-208 or Fisher Scientific, cat. no. FB102)
- Falcon FACS tubes with cell strainer (Corning, cat. no. 352235)
- Cell strainers, 100 μ m (Fisher Scientific, cat. no. 22-363-549)
- Long transfer pipettes (Fisher Scientific, cat. no. 13-711-39)
- BD FACS Sort II Aria (BD, cat. no. special order)
- FlowJo software (FlowJo, <https://www.flowjo.com/solutions/flowjo/downloads>)
- 50-ml Falcon conical tubes (Fisher Scientific, cat. no. 352070)
- 15-ml Falcon conical tubes (Fisher Scientific, cat. no. 352096)
- 4D Nucleofector Core Unit and X Unit (Lonza, cat. nos. AAF-1002B and AAF-1002X)
- Amaxa Nucleofector IIb (Lonza, cat. no. AAB-1001)
- Sorvall Legend XTR Centrifuge (Fisher Scientific, cat. no. 75004521)
- Faxitron Cabinet X-ray System (Faxitron, model no. 43855F)
- Extra Fine Bonn Scissors (FST, cat. no. 14084-08)
- Disposable scalpel blades, sterile, blade no. 22 (VWR, cat. no. 21909-626)
- High-precision no. 4 style scalpel handle (Fisher Scientific, cat. no. 12-000-164)
- Straight medium-point forceps (Fisher Scientific, cat. no. 16-100-106)
- MACS CD34 MicroBead Kit, human (Miteneyi, cat. no. 130-046-702)
- Heracell CO₂ incubator capable of controlling oxygen concentration (Fisher Scientific, cat. no. 51026282)
- C1000 Touch Thermal Cycler with Dual 48/48 Fast Reaction Module (Bio-Rad, cat. no. 1851148)
- Thermo Scientific MaxQ 5000 shaking incubator (Fisher Scientific, cat. no. SHKE5000)
- Precision general-purpose water bath (Fisher Scientific, cat. no. 2853)
- Heating block (Fisher Scientific, cat. no. 88870002)
- NanoDrop One (Fisher Scientific, cat. no. ND-ONE-W)
- LightCycler 480 Instrument II (Roche, cat. no. 05015243001)
- 0.22- μ m Vacuum filter (EMD Millipore, cat. no. SCGP00525)

REAGENT SETUP

K562 electroporation solutions—Solution I: Dissolve 2 g of ATP-disodium salt and 1.2 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 10 ml of nuclease-free ultrapure water, filter-sterilize, make 20- μl aliquots, and store them at -20°C for up to 2 months. Solution II: Dissolve 6 g of KH_2PO_4 , 0.6 g of NaHCO_3 , and 0.2 g of glucose in 500 ml of nuclease-free ultrapure water, adjust the pH to 7.4, filter-sterilize, make 1-ml aliquots, and store them at 4°C for up to 3 months. Mix 20 μl of solution I with 1 ml of solution II. This makes enough K562 electroporation solution for ten electroporations (100 μl each).

1 M electroporation solution—The 1 M electroporation solution is 5 mM KCl, 15 mM MgCl_2 , 120 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2, and 50 mM mannitol. Store aliquots at -20°C for up to 1 year⁴⁸.

Human engraftment antibody cocktail—Prepare a master mix of the following antibody volumes for 1 $\times$ samples: huCD45 (2 μl), CD33 (2 μl), mTer119 (0.25 μl), mCD45.1 (0.5 μl), CD19 (10 μl), HLA-A/B/C (1 μl), and if using *tNGFR* as reporter gene, include CD271 (5 μl). Store at 4°C in the dark for up to 24 h. Suspend the cell pellet in 100 μl of FACS buffer and add 15.75 μl (20.75 μl when including CD271) of the human engraftment antibody cocktail. ▲ **CRITICAL** Although these are the guidelines that we follow, the optimal concentration of each antibody should be empirically determined because of lot-to-lot variation.

In vivo HSC antibody cocktail—Prepare a master mix of the following antibody volumes for 1 $\times$ samples: CD90 (2 μl), CD10 (5 μl), CD38 (1 μl), CD34 (2 μl), CD45RA (2.5 μl), CD45.1 (1 μl), and if using *tNGFR* as reporter gene, include CD271 (5 μl). Store at 4°C in the dark for up to 24 h. Suspend the cell pellet in 100 μl and add 13.5 μl (18.5 μl when including CD271) of the *in vivo* HSC antibody master mix. ▲ **CRITICAL** Although these are the guidelines that we follow, the optimal concentration of each antibody should be empirically determined because of lot-to-lot variation.

In vitro HSC antibody cocktail—Prepare a master mix of the following antibody volumes for 1 $\times$ samples: CD34 (2 μl), CD45RA (2.5 μl), CD90 (2 μl), CD38 (1 μl), CD123 (5 μl), Lineage cocktail, 1 μl of each (CD2, CD3, CD4, CD8, CD16, CD19, CD20, CD56, CD235a (0.25 μl), and CD14), and if *tNGFR* is used as reporter gene, include CD271 (5 μl). Store at 4°C in the dark for up to 24 h. Suspend the cell pellet in 100 μl and add 21.75 μl of *in vitro* HSC antibody master mix (26.75 μl if including CD271). ▲ **CRITICAL** Although these are the guidelines that we follow, the optimal concentration of each antibody should be empirically determined because of lot-to-lot variation.

HSPC cytokine-rich medium—Culture all $\text{CD}34^+$ HSPCs in Steriflip-filtered StemSpan SFEM II supplemented with SCF (100 ng/ml), TPO (100 ng/ml), Flt3L (100 ng/ml), IL-6 (100 ng/ml), UM171 (35 nM), 20 mg/ml streptomycin, 20 U/ml penicillin, and SR1 (StemRegenin1; 0.75 μM). Store at 4°C for up to 1 week after preparation.

FACS buffer—FACS buffer is 1× PBS supplemented with 2% (vol/vol) FBS and 2 mM EDTA. Store at 4 °C for up to 2 months.

PROCEDURE

Preculture of CD34⁺ HSPCs ● TIMING 30 min–1 h hands-on; ~2 d of culture

- 1| After HSPC isolation or thaw, seed the cells in HSPC cytokine-rich medium (Reagent Setup) at a density of 0.25–0.5E6 cells/ml and culture at 37 °C, 5% CO₂, and 5% O₂.

▲ **CRITICAL STEP** Cells should be cultured in a low-oxygen incubator (5% O₂) for maintenance and expansion of LT-HSCs^{49,50}.
- 2| Culture the cells for 2 d (36–48 h). If the culture reaches 1 × 10⁶ cells/ml, split to 0.25–0.5 × 10⁶ cells/ml for optimal cell growth and viability, which also promotes high frequencies of HR.

Electroporation and transduction of cells ● TIMING 1–2 h hands-on; 2–3 d of culture

- 3| On Day 2 after thaw/isolation, count the number of viable cells using a hemocytometer and Trypan blue dye exclusion⁵¹.

▲ **CRITICAL STEP** Cells should be > 80% viable and may have expanded up to threefold depending on HSPC source. Cells with lower viability may be used, but this can negatively affect the gene-editing frequencies. We have electroporated as few as 10,000 cells using the Lonza 4D device with nucleocuvette strips that use electroporation volumes of 20 µl, but ideally use 100,000–1,000,000 cells per condition. When using electroporation cuvettes that hold 100 µl of cell suspension, we recommend electroporation of at least 250,000 cells and preferentially 500,000–1,000,000 cells (with a maximum of 5,000,000).
- 4| Prewarm an aliquot of HSPC cytokine-rich medium to 37 °C.
- 5| If using Lonza Nucleofection solutions, prepare solutions by mixing the entire supplement to the Nucleofector Solution (supplemented solution is active for 3 months). Alternatively, use the 1 M electroporation solution described in the Reagent Setup section. Allow the electroporation solution to equilibrate to room temperature.
- 6| Start the electroporation device and prepare the appropriate Nucleofector program. For the Lonza 4D device, we recommend the DZ100 program, and for the Lonza Iib, we recommend program U-014 for human CD34⁺ HSPCs.

▲ **CRITICAL STEP** These devices are interchangeable. The 4D device allows researchers to nucleofect small numbers of cells (< 100,000) in the nucleocuvette strip format (which can be beneficial when testing multiple experimental groups), whereas both the 4D and Iib nucleofectors handle electroporation cuvettes that require 500,000–1,000,000 cells for optimal transfection efficiencies.

- 7| Complex Cas9 protein with synthetic and chemically modified sgRNA (see the **Supplementary Methods** for identification, design, and production of sgRNA). Per 500,000–1,000,000 cells using nucleofection cuvettes: in a PCR tube, mix 15 µg of Cas9 protein with 8 µg of sgRNA (molar ratio of 1:2.5). For the Lonza 4D nucleocuvette strips, scale down fivefold. Mix well, centrifuge briefly (2,000g, 25 °C, 5 s), and complex by incubation in a thermocycler at 25 °C for 10 min. Store at 4 °C until use (up to 4 h) or on ice.
- ▲ **CRITICAL STEP** Higher amounts of Cas9 protein can sometimes result in higher editing frequencies (must be determined empirically). The molar ratio of Cas9 protein to sgRNA can range from 1:2.5 to 1:5, but we have found that 1:2.5 is the most cost-effective ratio, as it minimizes the amount of synthetic sgRNA used.
- ▲ **CRITICAL STEP** Use stocks of Cas9 and sgRNA that are at high enough concentrations so that the combined volume does not exceed 20 µl (20% of the volume of the electroporation solution).
- 8| Meanwhile, pellet the desired number of cells (500,000–1,000,000 per electroporation) by centrifugation at room temperature, 300g for 5 min.
- ▲ **CRITICAL STEP** We recommend including the following minimum control samples in a targeting experiment: mock-electroporated cells receiving no AAV (Mock), mock-electroporated cells receiving AAV (AAV only), RNP-electroporated cells receiving no AAV (RNP only), and RNP-electroporated cells receiving AAV (RNP + AAV).
- ▲ **CRITICAL STEP** We recommend empirically determining the number of cells to use per 15–30 µg of Cas9 protein (resulting in a final concentration of 150–300 µg/ml after Step 10 below), but we have found that high frequencies of editing can be achieved with cell concentrations ranging from 1 to 200 million cells/ml.
- 9| Remove the supernatant completely and resuspend the cell pellet in an appropriate volume of electroporation solution (100 µl per electroporation). Pipette up and down carefully to make a single-cell suspension.
- 10| Per electroporation, transfer 100 µl of cell suspension from Step 9 to the PCR tube with complexed Cas9 RNP from Step 7. Mix by pipetting gently and avoiding the formation of air bubbles. Transfer the entire volume to the electroporation cuvette and gently tap the cuvette to make sure that the sample covers the bottom of the cuvette.
- 11| Attach the lid to the cuvette, transfer it to the electroporation device, and electroporate the cells with the appropriate program selected in Step 6.
- ▲ **CRITICAL STEP** Do not allow the cells to sit in the electroporation solution for extended periods of time (> 15 min). This can lead to reductions in viability and reduced electroporation efficiencies.

- 12| Directly after electroporation, add prewarmed HSPC cytokine-rich medium to the cuvette for a final cell density of 1×10^6 cells/ml. Mix very gently with a sterile transfer pipette and transfer the cells to a 24-well plate. Optionally, cells can be left to rest in the incubator for up to 20 min before transduction.
- 13| (Optional) Transfer 50,000 cells to a 96-well plate and adjust the cell density to 0.25×10^6 cells/ml using prewarmed HSPC cytokine-rich medium. This will serve as an 'RNP-only' sample to measure INDEL frequencies without the presence of the AAV6 donor.
- 14| Transduce the cells from Step 12 by adding AAV6 donor vector (prepared as described in the **Supplementary Methods**). Adjust the cell density with prewarmed HSPC cytokine-rich medium, so that the cells are transduced at a density of $0.25\text{--}0.5 \times 10^6$ cells/ml overnight and not for > 24 h.
- ▲ **CRITICAL STEP** The volume of AAV6 donor vector should not exceed 20% of the total culture volume. If using AAV preparations that have suboptimal titers, add HSPC cytokine-rich medium to keep the AAV volume at $< 20\%$. However, we recommend not transducing at lower cell densities than 0.1×10^6 cells/ml, as this may compromise transduction efficiencies.
- ▲ **CRITICAL STEP** Transduce with rAAV6 within 1 h of electroporation, and ideally within 20 min of electroporation, to achieve high levels of HR.
- 15| After overnight transduction or 24 h after transduction, add prewarmed HSPC cytokine-rich medium to the cells to reach a density of $0.25\text{--}0.5 \times 10^6$ cells/ml and transfer the cells to an appropriate cell culture dish.
- 16| Culture the cells for 2–3 d while not exceeding cell densities of 1×10^6 cells/ml. Depending on the transgene, promoter, and targeted locus, expression of the transgene can reach a maximum between Days 2 and 3 after electroporation and transduction.

Measurement of HR frequencies and confirmation of stem and progenitor phenotype ●

TIMING ~1–2 h

▲ **CRITICAL** Following Steps 17–21, initial experiments for a particular target locus should assess which day the frequency of cells that express high levels of the reporter gene peaks (see Experimental design). This typically occurs between Days 2 and 3 after electroporation and transduction (Step 16). For all experiments, and particularly for transplantation studies, we recommend assessing at least the CD34 surface expression or a more elaborate set of LT-HSC phenotypic markers by flow cytometry. Within 4–5 d after cell isolation/thaw, cells should be at $> 90\%$ CD34⁺.

- 17| Pellet at least 50,000 cells from each sample from Step 16 in two FACS tubes at room temperature, 300g for 5 min.
- 18| Aspirate the supernatant and resuspend in 100 µl of staining buffer.

- 19| Add antibody cocktail depending on which marker(s) to assess. Use option A to stain for HSPCs by CD34 expression or option B to stain for LT-HSCs and multipotent progenitors (MPPs).

(A) Staining for HSPCs by CD34 expression

- i. Add the CD34 antibody (and anti-NGFR antibody if used as reporter) to only one of the two tubes (the unstained CD34 tube will serve as negative control in flow cytometric analyses). Mix well and incubate on ice protected from light for 30 min. Optionally, include a sample stained with an isotype control antibody.

▲ **CRITICAL STEP** Include a mock-electroporated sample with and without AAV donor for gating reporter⁺ and reporter^{high} cells, respectively (Fig. 2).

(B) Staining for LT-HSCs and MPPs

- i. It is possible to assess HR frequencies in CD34⁺ subpopulations: HSCs (Lin⁻/CD34⁺/CD38⁻/CD45RA⁻/CD90⁺), MPPs (Lin⁻/CD34⁺/CD38⁻/CD45RA⁻/CD90⁻), lymphoid-primed multipotent progenitors (LMPPs; Lin⁻, CD34⁺, CD38⁻, CD90⁻, and CD45RA⁺), common myeloid progenitors (CMPs; Lin⁻/CD34⁺/CD38⁺/CD45RA⁻/CD123⁺), granulocyte–macrophage progenitors (GMPs; Lin⁻/CD34⁺/CD38⁺/CD45RA⁺/CD123⁺), and megakaryocyte–erythrocyte progenitors (MEPs; Lin⁻/CD34⁺/CD38⁺/CD45RA⁻/CD123⁻). Representative FACS plots of subpopulations are depicted in **Supplementary Figure 2** as well as in Figure 5f in Reinisch *et al.*⁵², Figure 1 in Majeti *et al.*²⁷, and Figure 2a in Psaila *et al.*⁵³. Add the *in vitro* HSC antibody cocktail, mix well, and incubate on ice in the dark for 30 min. Optionally, include samples stained with isotype control antibodies.

▲ **CRITICAL STEP** Include a mock-electroporated sample with and without AAV donor for gating reporter⁺ and reporter^{high} cells, respectively.

- 20| Fill the FACS tube with ice-cold staining buffer, pellet at 4 °C, 300g for 5 min, aspirate the supernatant, and resuspend in 200 µl of staining buffer with 1 µg/ml propidium iodide. Keep on ice in the dark until analysis.

▲ **CRITICAL STEP** Cells should be analyzed as soon as possible for best results, but can be stored for at least 4 h.

- 21| Analyze on a flow cytometer, e.g., a FACS Aria (BD) using appropriate compensation controls and cytometer settings. Representative resulting FACS plots are shown in Figure 2 and **Supplementary Figure 1**. For the HSC antibody panel, use the gating strategy outlined in **Supplementary Figure 2** or in Figure 1 in Park *et al.*⁴⁶. Optionally, sort different populations of CD34⁺ cells, e.g., LT-HSCs and MPPs, from freshly purified CD34s and then subject to genome-editing to obtain HR frequencies in hematopoietic cells enriched for primitiveness (see **Supplementary Figure 2** for representative FACS plots and gating scheme).

? TROUBLESHOOTING

Performing CFU assay on targeted cells ● TIMING 3–6 h hands-on; 12–16 d of culture

- 22| Thaw MethoCult medium at room temperature or overnight at 4 °C.
▲ **CRITICAL STEP** Do not thaw MethoCult medium at 37 °C.
- 23| Shake the MethoCult medium vigorously for 1 min and let it stand for at least 5 min for bubbles to disappear.
- 24| With a 10-ml Luer lock syringe attached to a 16-gauge blunt-end needle, dispense one drop (~100 µl) of MethoCult medium into each well of a flat-bottom 96-well plate, leaving all outer wells empty.
- 25| Fill the outer wells with either sterile PBS or water and put the plate into a 37 °C incubator.
- 26| Prepare a fluorescence-activated cell sorter (FACS, e.g., a FACS Aria) for aseptic single-cell sorting into 96-well plates.
- 27| Sort the targeted population of CD34⁺ HSPCs (from Step 16 as described in Steps 17–21) (reporter^{high} population) one cell per well into the 60 wells with MethoCult medium from Step 24. In addition, sort the cells from a mock-electroporated sample, which will serve as a reference for total colony count and distribution of progenitor types.
- 28| Centrifuge the 96-well plate at room temperature, 300g for 5 min.
- 29| Incubate at 37 °C, 5% CO₂, and 5% O₂.
- 30| 12–16 d after sorting, use a high-quality inverted microscope equipped to count colonies and score them according to morphology. Extensive guidelines on CFU scoring, as well as representative colony images can be found in the technical manual for MethoCult.

? TROUBLESHOOTING

Extraction of genomic DNA from methylcellulose-derived colonies ● TIMING 1–2 h

- 31| Circle the wells from Step 30 that contain colonies. Fill the wells with PBS and pipette up and down to dilute the viscous methylcellulose.
- 32| Transfer the contents of each circled well to a well of a U-bottom 96-well plate and pellet the cells at room temperature, 300g for 5 min.
- 33| Invert the plate on a tissue towel to remove the supernatant, resuspend the cells in 250 µl of PBS, and pellet at room temperature, 300g for 5 min.
- 34| Invert the plate on a tissue towel to remove the supernatant, add 25 µl of QuickExtract DNA Extraction Solution per well, and pipette up and down to resuspend the cells.
- 35| Transfer the cells to a PCR plate and incubate at 60 °C for 10 min, and then at 100 °C for 10 min.

■ **PAUSE POINT** Colony-derived genomic DNA can be stored at -20°C for at least 1 year.

PCR-based genotyping of methylcellulose-derived clones with targeted integration ●

TIMING 4–6 h

- 36| Design the primers as outlined in the Experimental design section and depicted in Figure 3.

▲ **CRITICAL STEP** It is important to follow general PCR primer design guidelines to increase the chances of identifying functional primers with high specificity; often, multiple primers will need to be tested.

- 37| To test if primers are functional and to find the optimal annealing temperature, use a thermocycler with gradient technology and pooled genomic DNA from at least ten clones as template DNA. Set up a PCR master mix for eight reactions (+ 1 extra):

Phusion PCR Master Mix (2×):	112.5 μl
Primers (25 μM stock)	$3 \times 4.5 \mu\text{l}$
Template DNA	9 μl
Nuclease-free water	90 μl
Total volume	225 μl

- 38| Distribute the PCR master mix between eight PCR tubes (25 μl per tube).
- 39| Run the PCR reactions on a thermocycler programmed with the following thermal cycling parameters:

Cycle number	Denature	Anneal	Extend	Final
1	98 $^{\circ}\text{C}$, 30 s			
2–36	98 $^{\circ}$, 30 s	Gradient from 57 to 72 $^{\circ}\text{C}$, 30 s	72 $^{\circ}\text{C}$, 30 s/kb	
37			72 $^{\circ}\text{C}$, 5 min	4 $^{\circ}\text{C}$, α

■ **PAUSE POINT** PCR reactions can be stored at -20°C for at least 1 year.

- 40| Run each of the 25- μl PCR products and the GeneRuler 100 bp Plus DNA Ladder on a 1% (wt/vol) agarose gel at 100 V.
- 41| When the yellow dye present in the green PCR buffer has migrated to the bottom of the gel, visualize the gel on a UV gel-imaging system.
- 42| Identify the annealing temperature with the highest PCR efficiency and specificity.

? TROUBLESHOOTING

- 43| For each of the colony-extracted genomic DNA samples from Step 35, repeat the PCR, following Steps 37–39 (scale the PCR master mix according to the total sample number) and using the optimal annealing temperature identified in Step 42, and run the PCR products on an agarose gel, following Steps 40–41.
- ▲ **CRITICAL STEP** Include PCR reactions with genomic DNA from a colony derived from mock-treated cells and a nontemplate control reaction.
- 44| Using a scalpel, excise the PCR fragments from the gel and gel-purify bands using the GeneJET Gel Extraction Kit and following the kit's instructions.
- 45| Sanger-sequence the purified PCR fragments using appropriate primers from the PCR reaction.
- 46| Align Sanger sequences with the reference sequence to validate on-target and seamless HR-mediated integration of the transgene cassette. Sanger sequencing of nonintegrated alleles may be performed for identification of INDELs. Note that it is possible (although infrequent) to obtain clones with incomplete HR events⁵⁴. These frequencies must be empirically determined and may be noticeable by changes in PCR band sizes.

Transplantation of gene-modified CD34⁺ HSPCs ● TIMING 3–10 h hands-on; 16 weeks of engraftment

- 47| 24 h before transplantation, sublethally irradiate the desired number of 6- to 8-week-old NSG mice (preferably female⁴⁷) with 200 cGy.
- 48| For transplantation of cells not enriched for targeting, skip to Step 53. Immediately before transplantation, phenotype cells from Step 16 for the presence of CD34, as described in Steps 17–21. For transplantation of enriched cells, prepare the cells as described in Steps 17–21 (prepare all the cells that are to be transplanted).
- ▲ **CRITICAL STEP** Transplant mock-electroporated cells into two to three mice as a positive control for engraftment and for setting the reporter⁺ gate during flow cytometric analysis of engraftment if using a reporter gene.
- 49| Prepare a FACS (e.g., a FACS Aria) for aseptic cell sorting into an appropriate-sized tube depending on the expected number of cells to be sorted.
- 50| Sort viable targeted cells (reporter^{high} as depicted in Fig. 2). Optionally, include a CD34 stain and sort for CD34⁺ cells into prechilled PBS or HSPC cytokine-rich medium and collection tube holders chilled with 4 °C recirculating water.
- 51| Test the sort purity by analyzing a small subset of the sorted cells (250–500 cells) on the FACS. The purity should be > 95%.
- 52| Using a hemocytometer and Trypan blue dye exclusion, count the number of sorted viable cells.
- 53| Pellet the desired number of cells at 4 °C, 300g for 5 min. Resuspend in 200 µl of PBS for intravenous transplantation or 25 µl of PBS for intrafemoral

transplantation. Keep the cells on ice until transplantation is performed. Note that enriched targeted HSPCs have fewer absolute HSCs because of the reduced HR efficiencies in long-term repopulating HSCs; therefore, infusing a greater number of enriched targeted CD34⁺ HSPCs will usually lead to higher levels of engraftment. For tail-vein injection of sorted reporter^{high} cells, we recommend transplanting at least 500,000 cells (cord blood–derived). For intrafemoral injections, we recommend transplantation of at least 50,000 cells (cord blood–derived). Cells derived from mobilized peripheral blood or bone marrow usually require higher cell numbers to reach the same engraftment levels as those of cord blood–derived cells because of the lower fraction of LT-HSCs in the CD34⁺ population.

- 54| Transplant the cells either intrafemorally or intravenously (by tail-vein injection) using an insulin syringe with a 27-gauge × 1/2-inch needle into the recipient mice irradiated 24 h before transplantation.

Terminal analysis of engraftment in NSG mice at week 16 ● TIMING 5–10 h

- 55| Euthanize the mice according to the institutionally approved protocol.
- 56| Analyze engraftment in the bone marrow (option A), in which human chimerism is highest (five to ten times higher), or, if desired, analyze engraftment in the blood (option B) or spleen (option C).

▲ **CRITICAL STEP** Include a mouse not transplanted with human cells as a negative control for human engraftment and for setting the human cell gate during flow cytometric analysis of engraftment.

(A) Analysis of engraftment in the bone marrow

- i. Dissect femurs, tibiae, humeri, pelvises, sternum, and vertebral column and remove the muscle and connective tissue. Transfer the bones to a mortar containing 5 ml of RPMI with 10% (vol/vol) FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 4 U/ml heparin, and 20 U/ml DNase.

▲ **CRITICAL STEP** Optionally, you could instead analyze the bone marrow only from femurs. Do this by holding femurs with tweezers and inserting a 27-gauge needle attached to a 5-ml syringe filled with the above RPMI buffer into the bone marrow and flushing the marrow out into a Petri dish. If doing this, pipette the marrow up and down, transfer to a FACS tube through a 45-μm filter, and proceed directly to Step 56B(v). Note that mice transplanted intrafemorally will display higher engraftment in the injected as compared with the noninjected femur, and we therefore recommend keeping them separate throughout the engraftment analyses.

- ii. Crush the bones using a pestle, and filter the liquid through a 100-μm filter into a 50-ml conical tube.

- iii. Add another 5 ml of RPMI containing 10% (vol/vol) FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 4 U/ml heparin, and 20 U/ml DNase to the mortar.
- iv. Repeat Step 56A(ii).
- v. Incubate the cell suspension in a 37 °C water bath for 10 min for DNase treatment.
- vi. Add 40 ml of FACS buffer to the 50-ml conical tube. Using a pipette filler, fill a 10-ml serological pipette with 14 ml of Ficoll-Paque Plus and place the pipette at the bottom of the 50-ml conical tube. Remove the pipet filler and let gravity underlay the Ficoll-Paque Plus beneath the cell suspension. With a thumb against the end of the pipette, gently remove it from the 50-ml conical tube. Some Ficoll-Paque Plus will be retained in the pipette.
- vii. Centrifuge for 25 min at 850g at room temperature with full acceleration but no brake.
- viii. After centrifugation, aspirate the upper layer, leaving the mononuclear cell layer undisturbed.
- ix. Using a transfer pipette, carefully transfer the mononuclear cells to a new 50-ml conical tube.
- x. Fill the 50-ml conical tube with ice-cold FACS buffer. Cells can be stored on ice for up to 4 h.

(B) Analysis of engraftment in the blood

- i. Using a 27-gauge insulin syringe, immediately after euthanasia, extract blood from the heart and transfer it to an Eppendorf tube containing 200 µl of PBS with 10 mM EDTA.
- ii. Add 500 µl of PBS with 2% (wt/vol) dextran to the blood sample and mix well.
- iii. Incubate at 37 °C for 30 min to sediment red blood cells. During incubation, the spleen can be processed following Step 56C(i–vii).
- iv. After 30-min incubation of the blood sample, transfer the leukocyte-rich plasma layer to a FACS tube while being careful to avoid disturbing the sedimented red blood cell layer.
- v. Fill the FACS tube with ice-cold FACS buffer and centrifuge at 4 °C, 300g for 5 min to pellet the cells.
- vi. Aspirate the supernatant and resuspend the cells in 500 µl of red blood cell lysis buffer to lyse residual red blood cells. Incubate on ice for 5 min.
- vii. Fill the FACS tube with ice-cold FACS buffer and store on ice for up to 4 h.

(C) Analysis of engraftment in the spleen

- i. Dissect the spleen from the mouse and transfer to a six-well plate with 4 ml of RPMI containing 10% (vol/vol) FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 4 U/ml heparin, and 20 U/ml DNase.

■ **PAUSE POINT** Spleens can be left in the RPMI solution on ice for several hours while dissecting bones (Step 56A).
- ii. Put the spleen on top of a 100-µm filter and put the filter against the RPMI solution in the same well. With the end of a syringe plunger, grind the spleen against the filter until it has disintegrated and the cells have passed through the filter mesh and into the RPMI solution.
- iii. Transfer the cells to a FACS tube through the 0.45-µm filter cap.
- iv. Incubate in a 37 °C water bath for 10 min for DNase treatment.
- v. Fill the FACS tube with ice-cold FACS buffer and centrifuge at 4 °C, 300g for 5 min to pellet the cells.
- vi. Aspirate the supernatant and resuspend the cells in 500 µl of red blood cell lysis buffer to lyse residual splenic red blood cells. Incubate on ice for 5 min.
- vii. Fill the FACS tube with ice-cold FACS buffer and store on ice for up to 4 h.
- 57| Pellet the cells in a FACS tube by centrifugation at 4 °C, 300g for 5 min. Aspirate the supernatant, which should leave ~100 µl of buffer in the tube.

▲ **CRITICAL STEP** As the bone marrow contains a vast number of cells, we recommend staining 1/10 (5 ml) of the cells extracted from the Ficoll density centrifugation. For the cells from the blood and spleen, all cells can be stained. The remaining cells from the bone marrow (45 ml) can optionally be used to identify targeted human CD34⁺ HSPCs in the mouse bone marrow (Step 63), to establish single-cell-derived methylcellulose clones from the targeted human CD34⁺ HSPCs in the mouse bone marrow (Step 64), and/or to perform transplantation into secondary recipient mice (Step 65).
- 55| Block Fc receptors by adding 5 µl of TruStain FcX, mix well, and incubate at room temperature for 5 min.
- 59| Add the human engraftment antibody cocktail, mix well, and incubate on ice in the dark for 30 min. Optionally, if the flow cytometer allows, the antibody panel can be expanded to stain for markers of hematopoietic lineages other than B cells and myeloid cells.
- 60| Fill the FACS tube with ice-cold FACS buffer and pellet the cells by centrifugation at 4 °C, 300g for 5 min.
- 61| Aspirate the supernatant and resuspend in 400 µl of ice-cold FACS buffer with 1 µg/ml propidium iodide.
- 62| Analyze on a flow cytometer, e.g., a FACS Aria with proper compensation settings and controls.

▲ CRITICAL STEP If detecting expression of a fluorescent protein, use unstained cells for compensation that express the fluorescent protein from the same promoter as used in the donor template. We routinely use either CD34⁺ HSPCs targeted with the same AAV6 donor or K562 cells electroporated with the donor plasmid 2 d in advance. Examples of the gating strategy for identifying human cells, human B and myeloid cells, as well as reporter⁺ cells (GFP⁺), can be seen in Figure 4 and **Supplementary Figure 3**.

? TROUBLESHOOTING

- 63| (Optional) If desired, analyze the presence of engrafted CD34⁺ human cells in the bone marrow of the primary recipients at week 16. Stain 5 ml of the cells from Step 56A(x) for HSPC markers (CD34⁺, CD38⁻, and CD10⁻) using the *in vivo* HSC antibody cocktail and following Steps 57–62. Note that CD10 negatively discriminates for immature B cells that are also CD34⁺.
- 64| (Optional) Establish single-cell-derived methylcellulose clones from CD34⁺ human cells in the bone marrow of the primary recipients at week 16. This enables precise clonal genotyping of the long-term repopulating cells. Single-cell sort stained cells from Step 63 (CD34⁺, CD38⁻, and CD10⁻) into 96-well plates with methylcellulose, as outlined in Steps 22–30. Extract gDNA as described in Steps 31–35 and perform PCR-based genotyping described in Steps 36–46.
- 65| (Optional) Perform secondary transplantations to assess true long-term hematopoietic reconstitution potential using the remaining bone marrow-derived mononuclear cells not stained for human engraftment in Step 56A(x). Irradiate NSG mice 24 h before (Step 47) and transplant according to Steps 48–54. Before pelleting and resuspension in Step 53, filter the cells through a 45-μm mesh. Alternatively, for low-engrafting mice, it is advised to pool MNCs and enrich for CD34⁺ cells using the MACS CD34 MicroBead Kit. We recommend transplanting 5–10 million mononuclear cells intravenously or 2 million mononuclear cells intrafemorally. Human engraftment in secondary mice should be analyzed at least 12 weeks after transplantation, following Steps 55–62 (Fig. 5).

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Steps 1 and 2, isolation/thawing and preculture of CD34⁺ cells: 30 min–1 h hands-on, 2 d of culture

Steps 3–16, electroporation and transduction of cells: 1–2 h hands-on, 2 to 3 d of culture

Steps 17–21, measurement of HR frequencies and confirmation of stem and progenitor phenotype: 1–2 h hands-on

Steps 22–30, performing of CFU assay on targeted cells: 3–6 h hands-on, 12 to 16 d of culture

Steps 31–35, extraction of genomic DNA from methylcellulose-derived colonies: 1–2 h hands-on

Steps 36–46, genotyping of clones for targeted integration using PCR: 4–6 h hands-on

Steps 47–54, transplantation of gene-modified CD34⁺ cells: 3–10 h hands-on, 16 weeks of engraftment

Steps 55–65, performing terminal analysis of engraftment in NSG mice: 5–10 h hands-on

Box 1, homologous recombination in primary human T cells: 6–8 h hands-on, 5–7 d of culture

ANTICIPATED RESULTS

Genome editing by HR in HSCs allows for the investigation of HSC gene function, as well as for the *ex vivo* correction of disease-causing mutations for the development of the next generation of gene and cell therapies. By combining the CRISPR/ Cas9 system delivered as RNPs with homologous donor delivery via rAAV6, we routinely achieve > 20% (and up to 77%) HR frequencies in CD34⁺ HSPCs *in vitro* at several human loci, and assess reporter^{high} expression via FACS after successful HR into the intended locus 1–3 d post electroporation. Figure 2 and **Supplementary Figure 1** display the expected results from a successful HR experiment with a reporter^{high} population present only when the CRISPR components are added to the cells. Importantly, sorting of this reporter^{high} HSPC population enriches for cells with targeted integration, as shown in Figure 2, and also circumvents potential inefficient HSC targeting by removing untargeted HSCs that can compete for reconstitution in the bone marrow. CFU assays generally result in 30–50% colony formation with > 60% of colonies being CFU-GM and < 5% being CFU-GEMM, but the results can vary depending on HSPC source. Upon transplantation into NSG mice, we generally observe human engraftment in all transplanted mice, and with transplantation of enriched HSPCs, we generally observe that > 80% of the human cells are reporter⁺. Figure 4 shows the expected results after flow cytometric analysis of bone marrow engrafted with transplanted human cells. We envision that this methodology will be widely used for studying HSPC biology and advancing the field of gene and cell therapy for blood and immune system disorders.

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Figure 1.

Schematic overviews of design strategies for different donor types. **(a)** A reporter gene expression cassette driving the expression of, for example, a fluorescent protein (FP) can be integrated site-specifically by homologous recombination. 400-bp homology arms (in gray) are split at the CRISPR/Cas9 cut site between nucleotides 17 and 18 of the sgRNA target site (target site depicted in white and PAM in red) and flank the transgene expression cassette (in blue). Upon HR, the cassette is integrated seamlessly into the cut site. **(b)** SNPs can be introduced ($X \rightarrow Y$ mutation depicted, blue and green, respectively) using a vector design with 1.2-kb homology arms that flank the region between the desired site of the SNP and the CRISPR/Cas9 cut site. In the donor, the region between the mutation and the cut site should be mutated (the example uses synonymous mutations denoted by asterisks; note that encoded amino acids are listed above nucleotides) to avoid early termination of the HR process due to full sequence homology. This also introduces necessary mutations to the PAM and sgRNA target site to prevent Cas9 recutting and INDEL formation after HR. **(c)** A cDNA sequence (in green, diverged using synonymous mutations, e.g., as depicted in **b**) can be introduced directly into the start codon (ATG, purple) of a gene to express a desired cDNA from the endogenously regulated expression elements. A separate expression cassette

(blue) can be included after the cDNA, encoding, for example, a fluorescent protein (FP), which allows tracking and/or enrichment of targeted cells. The cDNA and reporter cassette are flanked by two 400-bp homology arms (gray) that flank the start codon and the CRISPR/Cas9 cut site. Seamless HR ensures that the cDNA is integrated in frame with the start codon. In an analogous manner, a 2A-cDNA cassette can be integrated immediately before the stop codon to link expression of a transgene to the expression of an endogenous gene. FP, fluorescent protein; LHA, left homology arm; pA, polyadenylation signal; RHA, right homology arm; SNP, single-nucleotide polymorphism.

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Figure 2.

Enrichment of gene-targeted CD34⁺ HSPCs using CRISPR/Cas9, AAV6, and FACS methodologies. (Left) Representative CD34⁺ HSPC FACS plots from day 4 post electroporation of Cas9 RNP and transduction of AAV6 (top) and transduction of AAV6 only (bottom) are shown, highlighting the generation of a reporter^{high} (GFP^{high}, shown in the red gate) population after the addition of Cas9 RNP (see also **Supplementary Figure 1** for FACS plots that include staining for CD34 expression). At day 4 post electroporation, targeted HSPCs from GFP^{high} (red), GFP^{low} (green), and GFP^{neg} (blue) fractions were sorted and cultured for 15 d while monitoring GFP expression by flow cytometry every 3 d (right). Note that the reporter^{high} population is > 96% reporter⁺ after 15 d in culture, highly indicative that this population is enriched for stable integration of the reporter cassette. neg, negative; SSC, side scatter. Image adapted with permission from ref. 15, Springer Nature.

Figure 3.

'In-Out' PCR strategy for genotyping on-target integration events in methylcellulose-derived colonies. (a) Schematic outlining the 'In-Out' PCR strategy for identifying on-target integration events in HSPCs. (Top) Primer design for the nontargeted allele using one primer that binds outside the left homology arm (LHA), and another primer that binds inside the right homology arm (RHA). Primers are depicted as red arrows. In the example presented, the PCR strategy will produce an 800-bp product (red) from a wild-type (WT)/INDEL allele. Note that the molecular weight of this band could be smaller or larger if an INDEL of substantial size is present. (Bottom) A targeted allele with a reporter cassette after CRISPR/Cas9 and AAV6-mediated homologous recombination. By using the same outside LHA (Out) primer as above, but with a reporter cassette-specific inside primer (In), this 'In-Out' PCR strategy will generate a 600-bp on-target integration-specific PCR product (purple). Primers are depicted as purple arrows. (b) A schematic representation of an agarose gel image showing the types of clonal integration events (when targeting an autosomal gene with one allele on each chromosome): WT or INDEL (800 bp, red), biallelic HR (600 bp, purple), and monoallelic HR (800 and 600 bp). Note that the presented PCR strategy is a three-primer PCR that analyzes all events in the same PCR. It is possible to separate the strategy into two PCR reactions. Furthermore, it is recommended to perform the same 'In-Out' strategy at the 3' end of the integration and, importantly, to Sanger-sequence PCR bands to confirm seamless HR at both ends.

Figure 4.

Gating strategy for engraftment analysis of NSG mice transplanted with targeted CD34⁺ HSPCs. Representative FACS plots showing gating scheme for identifying human cells (huCD45/HLA-ABC double positive) in the bone marrow of transplanted mice analyzed on a FACS Aria system with data analysis using the FlowJo software. **(a)** Representative FACS plot from the analysis of the bone marrow of a mouse not transplanted with human cells. The position of a human population is depicted in the red gate, which is transferred from **b**. The frequency of false-positive or contaminating human cells in this example is 0.001%. **(b)** Representative FACS plot from the analysis of bone marrow from a mouse transplanted with mock-electroporated cells. The left plot shows engraftment rates (2.2%) with human cells entailed in the red gate (positive for both huCD45 and HLA-ABC). The right plot shows GFP fluorescence of the human cells. A GFP⁺ gate is shown in green. **(c)** Representative FACS plot from the analysis of bone marrow from a mouse transplanted with RNP + AAV-treated cells (bulk). Human engraftment (red gate) and the frequency of targeted cells (GFP⁺, green gate) in the total human population are gated as in **a** and **b**. Furthermore, the bilineage potential (lymphoid and myeloid) of the engrafted human cells is analyzed by the presence of B cells (CD19⁺, lymphoid, blue gate) and myeloid cells (CD33⁺, purple gate), within which the frequency of targeted cells (GFP⁺) can also be quantified. **(d)** Representative FACS plot from the analysis of bone marrow from a mouse transplanted with RNP + AAV-treated cells sorted for GFP^{high} expression immediately before transplantation. The gating scheme is identical to that described above for **c**. See **Supplementary Figure 3** for the gating strategy upstream to the shown FACS plots. The experimental protocol was approved by Stanford University's Administrative Panel on Laboratory Animal Care. Image adapted with permission from ref. 15, Springer Nature.

Figure 5.

Flowchart for genome editing of CD34⁺ HSPCs and analysis of *in vitro* and *in vivo* HR frequencies. CD34⁺ cells are purified from fresh hematopoietic tissues or thawed from frozen stocks, and then stimulated with cytokines to preserve stemness, and promote cycling, and survival *in vitro*. Stimulated CD34⁺ HSPCs are then targeted using CRISPR/Cas9 and AAV6, HR efficiencies are evaluated *in vitro*, and CFU assays and NSG repopulation studies are initiated. Finally, *in vivo* HR frequencies in HSCs are evaluated by analysis of long-term engraftment of human cells in the bone marrow of NSG mice. Note that secondary transplants are recommended to confirm HR in true long-term HSCs.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
21	Low AAV6 transduction efficiencies	Low titer	Check transfection efficiency of HEK293T cells using a GFP plasmid control Check ITR integrity by performing Step 10 of the 'Cloning AAV donor plasmid' section of the Supplementary Methods
		Inaccurately measured titer	Retiter AAV6 (you can also use qPCR primers or a probe specific for the gene insert). Perform functional titration with AAV dose escalation to determine the MOI with the highest transduction level
		Weak promoter	Promoter may not be active in HSPCs. Switch to a stronger promoter such as SFFV or UbC
		Cas9 RNP not electroporated	For some promoters, episomal expression is low or nondetectable. When Cas9 is electroporated and a transgene is inserted by HR, the expression shift (Fig. 2) will lead to detectable expression
		Not using correct helper plasmid	There have been some reports of AAV6 not transducing HSPCs well, and we speculate that different subtypes of AAV6 exist. Contact the Russell lab at the University of Washington to acquire pDGM6 plasmid via Material Transfer Agreement. Alternatively, use the pDP6 plasmid from Plasmid Factory (cat. no. PF436)
	High cellular toxicity	Total volume of AAV6 exceeds 20% of culture volume	Add HSPC cytokine-rich medium to dilute AAV6 to 20% of the total volume (Step 14) or remake AAV6 to achieve a high titer ($>5 \times 10^{11}$ vg per ml). Alternatively, concentrate AAV6 using Vivaspin 15R concentrator (Vivaproducts, cat. no. VIVAVS15RH21)
		Cells were cultured at too high a density	Make sure that cells are split when reaching a density of 1×10^6 per ml
	Low CD34 ⁺ frequency in treated HSPCs	High cellular toxicity	See advice above for high cellular toxicity
		Poor isolation of CD34 ⁺ HSPCs	Be sure to follow the manufacturer's protocol for CD34 ⁺ isolation
	No or low reporter ^{high} HSPC population 2–4 days post electroporation and transduction	Low AAV transduction efficiencies	See advice above for high cellular toxicity
Inadequate electroporation of RNP complexes		Measure INDEL frequencies 4 d after electroporation in 'RNP-only' sample to confirm efficient electroporation and activity of Cas9 RNP. Alternatively, confirm efficient electroporation using GFP mRNA (TriLink Biotechnologies, cat. no. L-6101)	
Low or failed homologous recombination		Sequence the target locus in recipient cells to confirm intact sgRNA target site and donor homology arms	
Cells were cultured at a density that was too high		Make sure that cells are split when reaching a density of 1×10^6 per ml	
30	Low colony count in CFU methylcellulose assay	High cellular toxicity	See advice above for high cellular toxicity
		Methylcellulose is dried out	Remember to add water to all outer wells of the 96-well plate
		Cell sorter was not configured correctly	Make sure that the drop delay is accurately set before sorting using BD FACS Accudrop beads Align the Automatic Cell Deposition Unit to sort directly into the middle wells of a 96-well test plate
42	No PCR band after genotyping of methylcellulose clones	PCR inhibitors in gDNA	Use less gDNA from the QuickExtract procedure in the PCR reaction or scale up the PCR reaction while using the same gDNA amount
		Suboptimal primer design	Redesign and test several primers using a gradient of different annealing temperatures (57–72 °C)

Step	Problem	Possible reason	Solution
	Low PCR sensitivity for one of the genotypes in the three-primer genotyping PCR	Suboptimal primer design	Redesign and test several primers using a gradient of different annealing temperatures (57–72 °C). Alternatively, use less of the unique primer for the easily detectable genotype, making sure that the overall sensitivity is not compromised
62	Low human chimerism	Low number of HSCs in transplanted cells	Transplant more cells. HSC numbers can vary markedly between HSPC donors. Switch to intrafemoral transplantation if doing i.v. injection. Switch to cord blood-derived HSPCs, which have higher frequencies of HSCs. Particularly for enriched targeted cells, the HSC frequency is lower than that in a nonenriched population because of lower HR frequencies in HSCs, and transplantation of enriched targeted cells therefore requires higher cell doses
		Transplantation injection failed	Practice the transplantation procedure using fresh nonmodified HSPCs, preferably from cord blood, as they have higher HSC numbers than cells from mobilized peripheral blood and bone marrow
		Suboptimal antibody staining	Use more antibody in the stain or determine optimal amounts by antibody titration experiments on freshly isolated PBMCs