Molecular Therapy
Methods & Clinical Development
Original Article

Intracellular RNase activity dampens zinc finger nuclease-mediated gene editing in hematopoietic stem and progenitor cells

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Over the past decade, numerous gene-editing platforms which alter host DNA in a highly specific and targeted fashion have been described. Two notable examples are zinc finger nucleases (ZFNs), the first gene-editing platform to be tested in clinical trials, and more recently, CRISPR/Cas9. Although CRISPR/Cas9 approaches have become arguably the most popular platform in the field, the therapeutic advantages and disadvantages of each strategy are only beginning to emerge. We have established a nonhuman primate (NHP) model that serves as a strong predictor of successful gene therapy and gene-editing approaches in humans; our recent work shows that ZFN-edited hematopoietic stem and progenitor cells (HSPCs) engraft at lower levels than CRISPR/Cas9-edited cells. Here, we investigate the mechanisms underlying this difference. We show that optimized culture conditions, including defined serum-free media, augment engraftment of gene-edited NHP HSPCs in a mouse xenograft model. Furthermore, we identify intracellular RNases as major barriers for mRNA-encoded nucleases relative to preformed enzymatically active CRISPR/Cas9 ribonucleoprotein (RNP) complexes. We conclude that CRISPR/Cas9 RNP gene editing is more stable and efficient than ZFN mRNA-based delivery and identify co-delivered RNase inhibitors as a strategy to enhance the expression of gene-editing proteins from mRNA intermediates.

INTRODUCTION

Over the past decade, rapid development of efficient genome-editing approaches has opened new doors in genetics and basic science fields,[1–3] brought new tools to the treatment of once-intractable infectious, genetic, and malignant pathologies, and even been applied as a diagnostic technique for the current COVID-19 pandemic.[4,5] Numerous and distinct gene-editing platforms have been developed, each of which share a common fundamental approach: recognition of a specific DNA sequence, followed by specific genetic (or epigenetic) modification at this site. Several approaches have been described. Engineered meganucleases integrate each of these functions into a single functional unit.[3] In contrast, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are made up of pairs of modular proteins containing separate DNA-binding and nuclease domains designed to flank the sequence of interest.[6,7] Finally, the clustered, regularly interspaced, short palindromic repeats (CRISPR) platform complexes a nonspecific recombinant bacterial nuclease protein, for example CRISPR-associated protein 9 (Cas9), with a single guide RNA (sgRNA) that directs the nuclease to a specific sequence of interest.[8,9] Whereas early gene-editing approaches were predicated on the ability to induce DNA double-strand breaks and repair by the error-prone nonhomologous end-joining pathway, recent strategies have sought to introduce similar changes either via single-stranded DNA "nicks"[10] or base-editing approaches that avoid DNA strand breaks entirely.[11,12]

Head-to-head comparisons of each gene-editing platform have focused on specificity, applying a wealth of assays designed to enumerate various off-target loci and the frequency of mutation at these sites relative to the targeted locus.[13–15] Although the ultimate goal of any gene-editing approach is to achieve 100% on-target fidelity, unintended off-target mutations may or may not constitute a major concern, depending on the locus that has been changed, and/or the *in vivo* persistence of the cell type in question.[16] The inherent immunogenicity of each platform is an increasingly relevant concern for clinical applications and has also been investigated by several groups. Because CRISPR-associated nucleases such as Cas9 are proteins derived from bacterial species found in nature, patients may have previously encountered these antigens and possess pre-existing anti-CRISPR immune responses that could limit safety and efficacy.[17,18] In this regard, an important advantage of engineered nucleases

Received 16 August 2021; accepted 19 November 2021;
https://doi.org/10.1016/j.omtm.2021.11.010.

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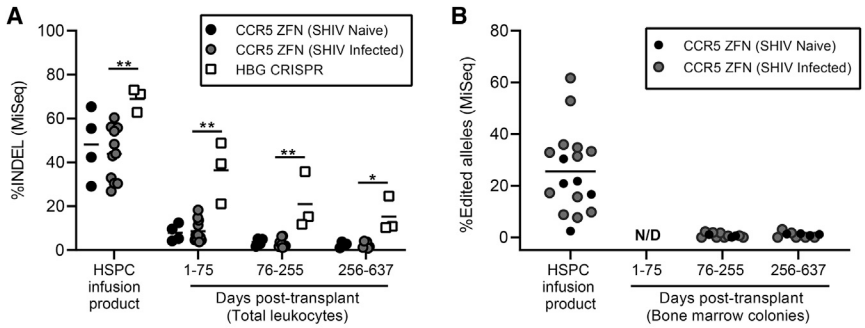

Figure 1. Engraftment kinetics of CCR5-ZFN-edited and HBG CRISPR-edited NHP HSPCs *in vivo*

(A) Average gene editing in peripheral blood. Statistical significance is denoted as *p < 0.05 and **p < 0.01. (B) Average CCR5 gene editing in bone marrow colony assays from pigtail macaques transplanted with ZFN-modified HSPCs. (C) Ratio of gene editing in HSPC infusion products *ex vivo* to post-transplantation peripheral blood or bone marrow total leukocytes. Data are derived from four SHIV-naive animals, 6–11 SHIV-infected animals (11 infusion products, of which *in vivo* samples were available from 6–11 over the course of up to 637 days' follow up), and three HBG CRISPR animals.

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Locus	Editing platform	Cell source	1-75 DPT	76-255 DPT	256-637 DPT
HBG	CRISPR-Cas9	Peripheral Blood	1.84	3.16	4.45
CCR5	ZFN	Peripheral Blood	5.32	14.26	20.15
CCR5	ZFN	Bone Marrow	N/D	35.93	27.55

such as ZFNs is that they should not be present in nature and therefore not susceptible to pre-existing immune responses.

Another key parameter is the efficiency with which each set of gene-editing machinery can be delivered to a given cell type, in this case, hematopoietic stem and progenitor cells (HSPCs). A major advantage of CRISPR platforms is the commercial availability of a nonvariant nuclease protein, which can be easily and rapidly complexed with a specific sgRNA *in vitro*. These so-called ribonucleoprotein (RNP) complexes are immediately enzymatically active and can be directly and efficiently electroporated into cells. Conversely, ZFNs, TALENs, and meganucleases must be specifically and carefully engineered for each targeted locus. To date, the availability of these tools as recombinant proteins has been extremely limited due to the need for defined protein manufacturing parameters, which often require adjustments on a molecule-specific basis. To overcome this limitation, ZFNs, TALENs, and meganucleases are most often delivered to cells via a messenger RNA (mRNA) intermediate. Although delivery of mRNA to various cell types such as HSPCs is usually quite efficient,[19] this strategy notably requires that the mRNA subsequently be translated and imported into the nucleus prior to genomic targeting.

To compare the ZFN mRNA and CRISPR RNP platforms, we focused on gene editing of the C-C motif chemokine receptor 5 (CCR5) locus, which is designed to protect cells against infection with human immunodeficiency virus 1 (HIV-1) and/or to serve as a safe harbor locus.[20,21] We have reported on HSPC as well T cell gene-editing approaches using several editing platforms and *in vivo* models,[22–27] namely, in nonhuman primates (NHPs) that are infected with an HIV-like virus, simian/human immunodeficiency virus (SHIV). Consistent with studies in mouse models[28–30] and in clinical trials (ClinicalTrials.gov NCT02500849), this approach should give rise to a repopulated, CCR5-null immune system that is resistant to infection, much like individuals carrying the naturally occurring CCR5Δ32 mutation.[31] Notably, allogeneic transplantation of CCR5Δ32 HSPCs has led to at least two cases of HIV cure to date.[32,33]

In our previous NHP studies, we found that CCR5 ZFN-edited autologous HSPCs engrafted, persisted, and gave rise to multilineage CCR5-edited progeny.[22] Although we did observe correlates of antiviral efficacy in these experiments,[24] we found that the level of editing in engrafted HSPCs and progeny *in vivo* was too low to support stable remission of viremia in the absence of suppressive antiretroviral therapy (ART). In separate and more recent NHP experiments, we have achieved significantly higher levels of persistence of edited cells using the CRISPR/Cas9 platform.[26] In this study, we addressed a key question arising from these data: why do CRISPR-edited cells engraft and persist more efficiently than ZFN-edited cells? We investigated two potential mechanisms: (1) *ex vivo* culture conditions that were designed to maximize ZFN gene-editing efficiency but may impair engraftment of these cells; and (2) the stability of preformed and enzymatically active CRISPR/Cas9 RNPs, relative to ZFN mRNA, which must be translated in order to generate enzymatically active ZFN proteins.

RESULTS

Decreased persistence of ZFN- versus CRISPR-edited HSPCs

Our previous studies with the ZFN editing platform demonstrated that despite efficient levels of CCR5 editing *ex vivo*, long-term engraftment of CCR5-edited NHP HSPCs in autologous hosts was low.[22,24] We first reanalyzed the *in vivo* data from one of our previously published studies[24] in which HSPCs were electroporated with ZFN-encoding mRNA and transplanted into either naive or SHIV-infected, ART-suppressed NHPs. To quantify the persistence of edited cells *in vivo*, we calculated the ratio of CCR5 gene editing in HSPC infusion products to the respective levels of CCR5-edited leukocytes in each transplanted animal following hematopoietic recovery. In both uninfected and SHIV-infected, ART-suppressed animals, we observed a similar decline in CCR5 gene editing *in vivo* versus *ex vivo*. Despite editing close to 45% of CCR5 alleles in HSPC infusion products, less than 5% of alleles were edited in circulating total leukocytes (Figure 1A); we observed similar trends in bone marrow colonies (Figure 1B). The ratio of *ex vivo*:*in vivo* CCR5 editing was as high as

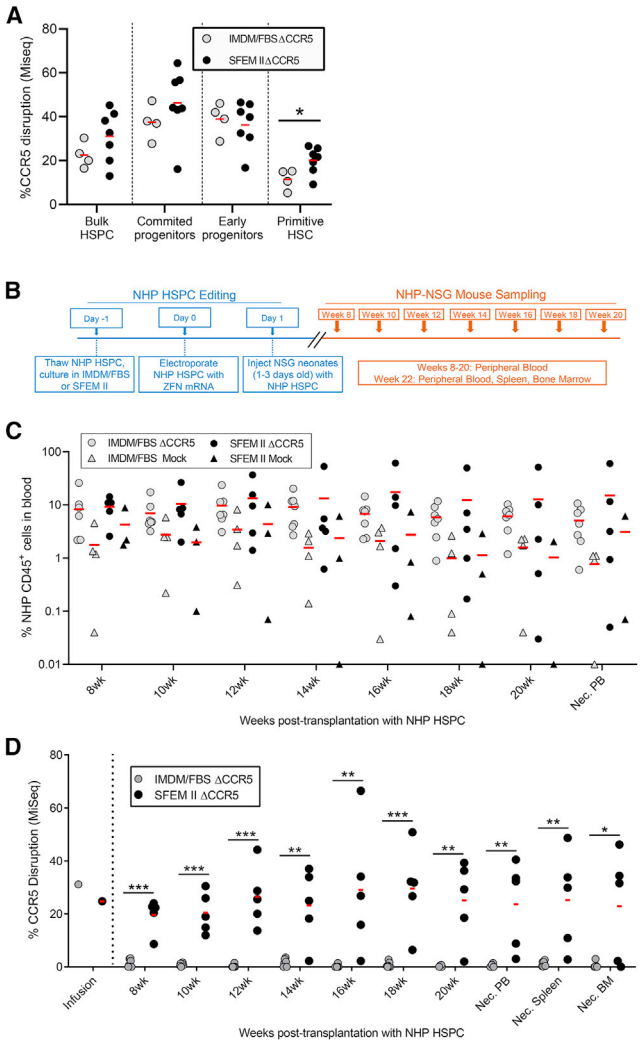

Figure 2. Improved engraftment of NHP HSPCs in humanized mice

(A) ZFN editing efficiency in bulk NHP HSPCs and HSPC subsets from 4–7 donors cultured in FBS-containing medium (gray) or serum-free medium (black). Bulk: $CD34^+$; Committed: $CD34^+CD90^-CD45RA^+$; Early: $CD34^+CD90^-CD45RA^-$; Primitive: $CD34^+CD90^+CD45RA^-$. (B) Schematic of the workflow for studying engraftment of CCR5-edited NHP $CD34^+$ cells in NSG mice. (C) CCR5 ZFN-edited or mock-edited NHP HSPCs were transplanted into NSG mice following culture in the indicated medium, followed by quantification of engraftment of NHP $CD45^+$ cells. Time points with undetectable NHP cells are plotted as 0.01% for graphing purposes. (D) CCR5 MiSeq analysis of total cells from the infusion product, peripheral blood between 8 and 20 weeks post transplant, and necropsy. Because NHP HSPCs were obtained as surplus from autologous transplantation experiments, we were limited to 1 NHP donor per experimental arm in (C) and (D). Statistical significance is denoted as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

20.15 in peripheral blood and 27.55 in bone marrow, consistent with poor engraftment and persistence of CCR5 ZFN-edited cells in both compartments (Figure 1C). In stark contrast, our more recent studies targeting the γ-globin promoter (HBG) with CRISPR/Cas9 ribonucleoprotein complexes[26] showed a significant improvement in the persistence of gene-edited cells (Figure 1A), resulting in a significantly lower *ex vivo*:*in vivo* editing ratio of 4.45 or less in peripheral blood (Figure 1C). Collectively, these results demonstrate that CCR5 ZFN-edited HSPCs engraft and persist less efficiently than HBG CRISPR RNP-edited HSPCs after autologous transplantation in NHPs.

Defined culture medium improves editing efficiency in true NHP hematopoietic stem cells

Our initial findings led us to investigate variables that could have destabilized HSPC gene-editing levels in our ZFN mRNA experiments relative to our CRISPR/Cas9 experiments. We first asked whether the culture media utilized during the manufacturing of CCR5-edited NHP HSPC products affected engraftment of these cells *in vivo*. In our initial CCR5 ZFN studies, cells were cultured in medium containing fetal bovine serum (FBS) with Iscove's modified Dulbecco's medium, referred to hereafter as IMDM/FBS. Consistent with more recent advancements in the field, our subsequent HBG CRISPR studies utilized defined serum-free medium, referred to hereafter as SFEM II. We first asked whether culture in IMDM/FBS versus SFEM II affected the efficiency of CCR5 editing in $CD34^+$ HSPC subpopulations, namely, in long-term engrafting hematopoietic stem cell (HSC) subsets that we have previously defined.[34] The protocol for culturing and editing NHP HSPCs in IMDM/FBS or SFEM II was matched to our previous *in vivo* experiments (Figure 1).[24] NHP HSPCs were isolated and cultured in either IMDM/FBS or SFEM II, electroporated with CCR5 ZFN mRNA, then sorted by fluorescence-activated cell sorting (FACS) 1 day after editing. Next-generation sequencing (NGS) was used to quantify CCR5 editing from total genomic DNA isolated from unsorted bulk HSPCs and the following sorted subsets: $CD34^+CD45^{dim}CD90^-CD45RA^+$ ("committed progenitors"), $CD34^+CD45^{dim}CD90^-CD45RA^-$ ("early progenitors"), and $CD34^+CD45^{dim}$ $CD90^+CD45RA^-$ ("primitive HSCs"). Although unchanged in bulk HSPCs, CCR5 editing was significantly more efficient in SFEM II medium compared with IMDM/FBS in the primitive HSC subset (Figure 2A).[34] Interestingly, media formulation affected neither the distribution of CD34 subsets (Figure S1), nor editing efficiency in the committed or early progenitor subsets, although each showed higher levels of editing than primitive HSCs, consistent with past reports[26] (Figure 2A). These data suggest that culture media formulation is an important factor in maximizing editing efficiency in primitive HSCs, the subset that makes the largest contribution to stable, long-term engraftment *in vivo*.

Defined culture medium improves engraftment of ZFN-edited NHP HSPCs

We next compared the engraftment of CCR5 ZFN-edited NHP HSPCs in serum-containing versus defined culture media, using a novel NHP-immunodeficient mouse xenograft model (Figure 2B). Edited cells were infused into neonatal NOD/SCID/IL2rγ^{null} (NSG) mice 1 day after editing, the same time frame in which HSPC subset sorting experiments were conducted in Figure 2A. Because NHP HSPCs were obtained as surplus from autologous transplantation experiments, we were limited to one NHP donor per experimental arm

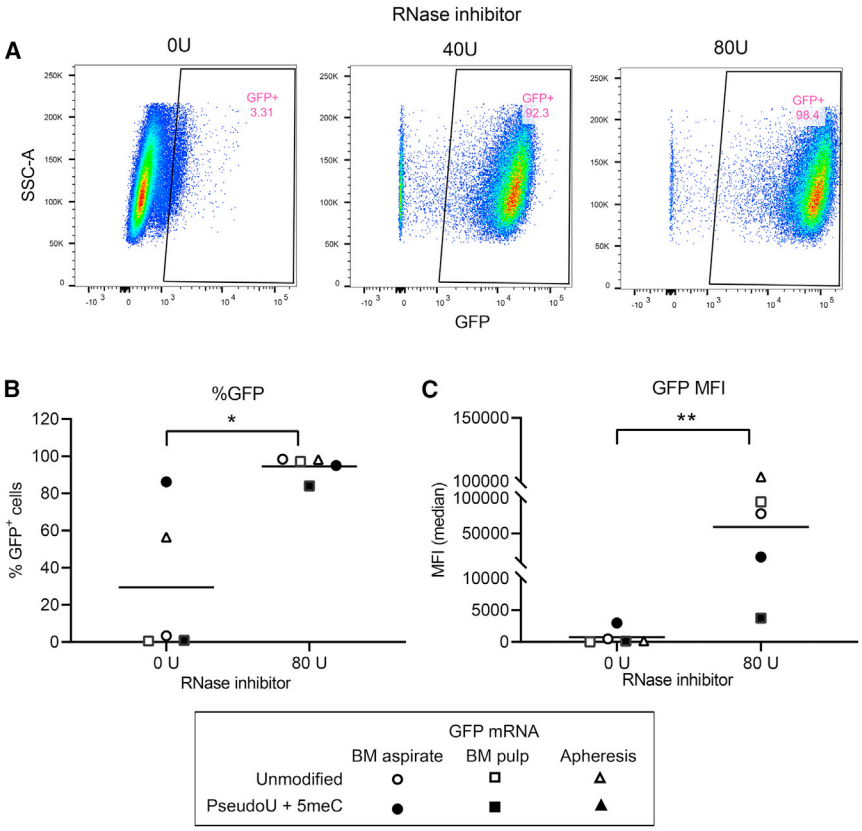

Figure 3. RNase inhibitors rescue exogenous mRNA expression in NHP HSPCs

(A) NHP HSPCs were electroporated with mRNA encoding GFP, with or without the indicated units of RNase inhibitor (RNasin) and assayed for GFP expression 24 h later by flow cytometry. (B) GFP expression with or without RNasin from three distinct NHP HSPC donors. Circles: bone marrow aspirate; squares: postmortem bone marrow pulp; triangles: mobilized apheresis. Open shapes: unmodified mRNA; filled shapes: mRNA modified with pseudo-uridine and 5-methylcytosine. (C) Median fluorescence intensity of data from (B). Statistical significance is denoted as *$p < 0.05$ and **$p < 0.01$.

(Figure S2). Unsurprisingly, engraftment of NHP HSPCs in NSG mice was lower than has previously been described for human HSPCs, likely due to species-specific differences in donor HSPCs.[35] Intriguingly, we saw higher levels of total NHP cell engraftment in CCR5-edited conditions versus mock conditions, independent of media formulation (Figure 2C). On the day of infusion (1 day post electroporation with ZFN mRNA), CCR5 editing was slightly higher in IMDM/FBS HSPCs (31.16%) than in SFEM II HSPCs (24.77%) (Figure 2D). In colony assays plated from each NHP CCR5-edited HSPC infusion product, the proportion of CCR5-edited alleles was comparable between media (Figure S2A), although biallelic editing was higher in the SFEM II condition (Figure S2B). We next measured editing levels in NSG mice that were infused with these NHP HSPCs, beginning 8 weeks post infusion. Across all time points, CCR5-edited HSPCs that were cultured in SFEM II supported significantly higher levels of CCR5 editing in peripheral blood than CCR5-edited, IMDM/FBS-cultured HSPCs (Figure 2D). At necropsy, we measured engraftment of edited cells in peripheral blood, spleen, and bone marrow. Again, we observed significantly higher levels of engraftment of CCR5-edited cells following SFEM II culture relative to IMDM/FBS. Strikingly, necropsy bone marrow analyzed in colony-forming assays did not yield any CCR5-edited colonies when collected from IMDM/FBS HSPC animals, whereas up to 25% biallelic editing was detected in SFEM II HSPC animals (Figure S2). A leading hypothesis derived from these findings is that serum-containing medium impairs the engraftment of CCR5-edited NHP HSPCs *in vivo*.

Intracellular RNases destabilize exogenous mRNA expression in NHP HSPCs

In addition to the impact of culture media, other parameters could additively contribute to stable engraftment of CCR5-edited NHP HSPCs. We hypothesized that delivery of an mRNA-encoded nuclease (ZFN) is less efficient than CRISPR/Cas9 RNP, in part because ZFN mRNA is susceptible to degradation by intracellular RNases. To test this, we first used a reporter mRNA encoding GFP as a surrogate for mRNA stability and expression in NHP HSPCs. We electroporated NHP HSPCs with GFP mRNA in the presence and absence of a recombinant RNase inhibitor protein cocktail ("RNasin") and measured GFP expression by flow cytometry 24 h later. Expression of GFP mRNA was rescued by RNasin (Figure 3A). Quantitative analyses from three independent NHP HSPC sources cultured in SFEM II medium (singlicate samples merged for comparison ± RNasin) revealed that co-delivery of GFP mRNA and RNasin led to a significant increase both in the number of GFP^+ cells (Figure 3B) and in GFP median fluorescence intensity (Figure 3C). Poorer expression of GFP mRNA correlated with use of unmodified mRNA molecules, whereas mRNA fully substituted with pseudo-uridine and 5-methylcytosine appeared nominally more stable (Figures 3B and 3C). Notably, cryopreserved NHP HSPCs displayed lower GFP expression than fresh NHP HSPCs in IMDM/FBS and SFEM II media, even with fully substituted mRNA (Figure S3). Taken together, these data clearly show that intracellular RNases represent an important barrier to efficient expression of exogenous mRNA in NHP HSPCs, which may be exacerbated by other factors including mRNA chemistry and fresh versus cryopreserved cell sources.

Inhibition of RNase activity in NHP HSPCs augments CCR5 editing efficiency

The primary goal of our study was to identify factors associated with inefficient ZFN editing of NHP HSPCs compared with CRISPR/Cas9

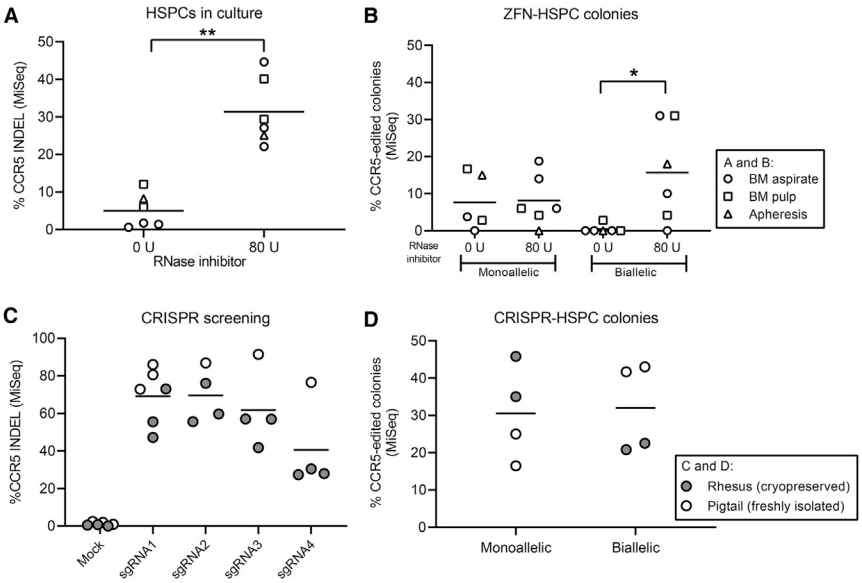

Figure 4. RNase inhibitors enhance CCR5 ZFN editing in NHP HSPCs

(A) NHP HSPCs (n = 3) derived from bone marrow aspirate (circles), postmortem bone marrow pulp (squares), and/or mobilized apheresis (triangles) were electroporated with unmodified mRNA encoding CCR5 ZFNs, with or without RNase inhibitor (RNasin), and assayed for CCR5 editing 5 days later. (B) Biallelic versus monoallelic editing in HSPC colonies derived from (A). (C) CCR5 editing in NHP HSPCs from 4–6 donors following electroporation with CCR5-targeted CRISPR ribonucleoprotein complexes (RNPs). Open circles: freshly isolated pigtail bone marrow cells; filled gray circle: cryopreserved rhesus bone marrow cells. (D) Biallelic versus monoallelic editing in HSPC colonies (n = 4) derived from sgRNA1-treated cells from (C). Statistical significance is denoted as *$p < 0.05$ and **$p < 0.01$.

RNP-based approaches. Hence, we next repeated experiments in Figure 3, except that instead of GFP mRNA, NHP CCR5 ZFN-encoding mRNA (without pseudo-uridine and 5-methylcytosine base substitution) was delivered with or without RNasin (Figures 4A and 4B). Consistent with our findings using a GFP mRNA reporter, we observed that co-delivery of RNasin enhanced ZFN-mediated CCR5 editing by an average of 6.3-fold (Figure 4A). The percentage of biallelic editing in HSPC-derived colonies was also significantly increased in the presence of RNasin (Figure 4B). The increase in biallelic but not monoallelic editing may suggest an "all-or-nothing" effect of stabilized ZFN mRNA expression on per-cell CCR5 editing in NHP HSPCs. Increased editing efficiency in the presence of RNase inhibitors was correlated with enhanced ZFN protein expression, as determined by western blotting with anti-Fok1 antibodies (Figure S4). Importantly, although RNasin enhanced ZFN-mediated gene editing, it also decreased HSPC colony-forming capacity (Figure S5A), suggesting that a more refined/targeted RNase inhibition approach will be necessary to maximize HSPC editing efficiency while minimizing toxicity. Nevertheless, our data provide proof-of-principle support for our hypothesis that intracellular RNases destabilize mRNA-based editing platforms in HSPCs, resulting in inefficient levels of CCR5 gene editing.

RNP-based CRISPR/Cas9 results in higher levels of CCR5 editing in HSPCs

Finally, we investigated whether use of preformed CCR5 CRISPR/Cas9 RNP complexes provides a feasible and more efficient alternative to mRNA-based gene-editing platforms, consistent with our more recent findings[26] (Figure 1). To directly compare NHP CCR5-targeted ZFN mRNA with CRISPR/Cas9 RNP at the same genetic locus, we first screened four unique sgRNAs targeting the NHP CCR5 locus (Figure S6 and Table S1). These experiments utilized both cryopreserved rhesus and freshly isolated pigtail macaque $CD34^+$ HSPCs to compare any differences in editing efficiency that could be attributed to the cell source (Figure S3). NHP CCR5 sgRNA1 and sgRNA2 showed the highest levels of gene editing (69.2% and 69.5%, respectively), followed by sgRNA3 (61.8%) and sgRNA4 (40.6%) (Figure 4C). CRISPR/Cas9 RNP-based CCR5 gene editing with sgRNA1 and sgRNA2 guides enabled NHP CCR5 editing levels twice that of ZFN mRNA, even in the presence of RNase inhibitors (Figure 4A); similar or greater increases in editing efficiency were observed when comparing mono- and biallelic editing in ZFN mRNA versus CRISPR RNP samples (Figure 4D). The slightly lower levels of editing seen with rhesus $CD34^+$ cells relative to pigtail $CD34^+$ cells can be attributed to the cryopreserved status of these cells and is unlikely to be due to a species-specific difference in the targeted CCR5 gene sequence, which was nearly identical between the two species (Figure S6B). Interestingly, CRISPR/Cas9 RNP editing reduced colony-forming capacity in NHP HSPCs comparably with ZFN editing with RNasin (Figure S5C), although neither ZFN mRNA (Figure S5B) nor CRISPR RNP (Figure S5D) measurably affected the distribution of various colony types. Collectively, these data show that CRISPR/Cas9 RNP-based gene editing is more efficient than ZFN mRNA-based approaches in NHP HSPCs, with or without RNase inhibitors.

DISCUSSION

Our study shows that intracellular RNases destabilize exogenous mRNA in HSPCs, hampering gene-editing efficiency for platforms such as ZFNs that rely on an mRNA intermediate to generate an enzymatically active editing protein. We provide proof of concept that recombinant RNase inhibitors can rescue ZFN mRNA expression and increase gene editing, although not to levels observed when using CRISPR/Cas9 RNP. In addition, we show that culture media formulation is a critical factor in maximizing editing efficiency in primitive HSC subsets, which makes the largest contribution to stable engraftment *in vivo*.

Long-term *in vivo* persistence of gene-edited HSPCs depends on at least two factors, each of which we addressed in this study: (1)

gene-editing efficiency in the infused HSPC product, specifically, the primitive HSC subset;[34] and (2) the engraftment potential of these cells following *ex vivo* manipulation. Using our well-established NHP model of HSPC gene therapy, we show first that culturing NHP HSPCs in serum-free medium significantly improves the engraftment of gene-edited cells *in vivo*. Interestingly, this approach also significantly augments editing efficiency in primitive HSC subsets. Second, we demonstrate that HSPC-associated RNase activity hinders the expression of exogenous mRNA. This represents an important limitation for any mRNA-based gene-editing approach relative to more stable RNP complexes made up of CRISPR sgRNA and Cas9 protein. The wide range of HSPC gene-editing efficiencies we observed following electroporation-based delivery of ZFN mRNA was an early indicator of potential limitations with this approach and is consistent with previous preclinical studies.[28] To our knowledge, our study is the first to characterize a mechanism underlying inefficient gene editing in HSPCs when using an mRNA-encoded nuclease, and is consistent with early results from recent clinical trials utilizing CRISPR/Cas9 RNP[36] versus ZFN mRNA (ClinicalTrials.gov identifier NCT03653247) to target the erythroid enhancer of the BCL11A gene as a treatment for hemoglobinopathies. In addition to RNase-dependent mechanisms (discussed below), we found that serum-containing media, the use of cryopreserved NHP HSPCs, and donor-specific phenomena could also contribute to the limited efficiency of mRNA-encoded gene-editing nucleases. The dramatic loss of ZFN-edited cells *in vivo* (40%–50% editing in infusion products, reduced to less than 5% *in vivo*) is consistent with a model in which serum-free media better support long-term HSC self-renewal properties and augment gene-editing efficiency in this subset. Importantly, we were unable to directly measure RNase levels in progenitor versus self-renewing HSC subpopulations, and hence cannot correlate RNase activity with reduced engraftment of long-term, ZFN-edited HSCs.

Co-delivering recombinant RNase inhibitors significantly improved the efficiency and reproducibility of exogenous mRNA expression in HSPCs. This finding is consistent with a model wherein RNases present in the cellular milieu rapidly degrade exogenous mRNAs following intracellular delivery, directly affecting expression and downstream function of the encoded transgene, in this case CCR5 ZFNs. The RNase inhibitors used in our study are broad spectrum, meaning that one or many RNase enzymes could contribute to this effect. Importantly, these inhibitors are expected to broadly perturb RNA homeostasis and RNA turnover, which could have triggered secondary impacts that influenced our findings. Previously, RNase inhibitors have been shown to improve the activity of a luciferase RNA reporter delivered via tail vein injection in mice,[37] although this strategy is designed to neutralize RNases found in circulating plasma. In contrast, perturbation of intracellular RNase expression patterns may have much more deleterious impacts on key HSPC functions such as long-term engraftment and self-renewal potential. For example, we found that while intracellular delivery of RNase inhibitors improved ZFN-dependent gene-editing efficiency, the colony-forming potential of these cells was reduced. Surprisingly, colony-forming capacity was similarly reduced following CRISPR/Cas9 treatment of HSPCs without RNase inhibitors, suggesting that toxicity in this assay may correlate with gene-editing efficiency, e.g., by triggering of DNA repair and cell death pathways. Extensive further studies will hence be required to characterize the feasibility of artificially overexpressing exogenous RNase inhibitors inside cells to boost intracellular mRNA expression for gene editing and other applications.

Data from at least one locus (NHP CCR5) clearly show that CRISPR/Cas9 RNPs consistently generate more indels than ZFN mRNA, even when the latter is augmented by RNase inhibitors. Of note, suboptimal ZFN engineering may have contributed to the effects we observe. Whether due to this limitation or mRNA instability, simply increasing the amount of ZFN mRNA delivered to a given number of HSPCs modestly increases editing efficiency.[28,29] However, the cost/benefit ratio of this strategy (i.e., costs for additional ZFN mRNA reagent relative to gain in editing and engraftment efficiency) requires closer examination, especially in the clinical setting. As an alternative to intracellular delivery of RNase inhibitors, our data also show that base-modified mRNA may partially stabilize ZFN-mediated gene editing. Previously, CRISPR/Cas9 delivered as "all RNA" (guide RNA plus Cas9 mRNA instead of Cas9 protein) have been similarly augmented.[38] Two head-to-head comparisons of CRISPR/Cas9 delivery by RNP versus all RNA affirm our conclusion that mRNA intermediates decrease editing efficiency.[39,40] Additional site-specific chemical modifications introduced to the three terminal nucleotides at the 5′ and 3′ ends of sgRNAs, along with uridine-depleted, base-modified Cas9 mRNA, reduced endogenous exonuclease degradation and innate immune stimulation and increased the editing efficiency in $CD34^+$ HSPCs.[39,41] Similar chemical modifications of base editor (BE) mRNA and guide RNA are necessary to mediate efficient editing of cells in culture.[11,42] Consistent with our findings, RNP-based delivery of guide RNA and purified BE protein more effectively overcame these limitations by bypassing the reliance on intracellular translation machinery to generate the enzymatically active nuclease complex.[43] These studies reinforce the notion that translation of exogenous mRNA introduces an additional step that is a barrier to optimal gene-editing efficiency, but can be at least partially counteracted by *cis* strategies to stabilize and reduce the immunogenicity of exogenous mRNAs. Surprisingly, a more recent BE study showed that BE mRNA is superior to BE RNP.[44] These contradictory findings most likely reflect differences in the targeted locus and/or the editing proteins in question, which would benefit from a larger-scale, multi-locus comparison that factors in potential differences between distinct editing platforms such as CRISPR/Cas9, CRISPR BE, and ZFN.

A common challenge associated with the delivery of exogenous mRNA in primary cells is the activation of innate immune pathways. RNA sensors such as Toll-like receptors directly sense these ligands, leading to release of pro-inflammatory cytokines and inhibition of translation.[41,45] As described above, multiple studies have incorporated naturally occurring nucleoside modifications such as pseudouridine, 5-methyluridine, and 5-methylcytidine in exogenously

manufactured mRNA to reduce its immunogenicity and decrease susceptibility to cleavage by ubiquitous cellular RNases.[46,47] However, consistent with our data, base-modified mRNA are only partially protected from anti-mRNA immune responses.[45] Previous studies suggest that some base-modified mRNAs can still lead to immune activation, due to structural signals such as double-strand RNA which activate RNase L, resulting in RNase degradation of these mRNAs.[45,48] An intriguing hypothesis related to our study is that growth factors, cytokines, or mitogens found in FBS could also modulate these pathways, i.e., that culture media formulation could affect exogenous mRNA stability following intracellular delivery. Collectively, these data suggest that endogenous immune responses likely contribute to decreased efficiency of gene editing by mRNA-encoded nucleases and the persistence of gene-edited cells *in vivo*, which can be only partially overcome through the use of base-modified mRNA.

In conclusion, we find that defined culture conditions are crucial to increase editing and engraftment of long-term HSCs in the NHP model, and that RNase-dependent degradation of exogenous mRNA, which limits ZFN-based strategies in NHP HSPCs, can be bypassed through the use of CRISPR/Cas9 RNP. While base-modified mRNA and/or co-delivery of recombinant, broad-spectrum RNase inhibitors partially rescue the expression of exogenous ZFN-encoding mRNAs, this approach remains inferior to delivery of CRISPR/Cas9 RNPs. The ZFN platform holds crucial advantages, including lack of pre-existing immunity, high fidelity, and a proven track record in the clinic. Although current mRNA-based delivery paradigms limit the efficiency of this platform, we believe that various adjustments could be made to improve the engraftment and long-term persistence of CCR5 ZFN-edited HSPCs *in vivo*.

MATERIALS AND METHODS

NHP studies

All experiments utilizing NHP HSPCs were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health ("the Guide") and were approved by the Institutional Animal Care and Use Committees of the Fred Hutchinson Cancer Research Center and University of Washington, Protocols #3235-01, -04, and -06. As described previously,[27] all animals were housed at and included in standard monitoring procedures prescribed by the Washington National Primate Research Center (WaNPRC). This included at least twice-daily observation by animal technicians for basic husbandry parameters as well as daily observation by a veterinary technician and/or veterinarian. Animals were housed in cages approved by the Guide and in accordance with Animal Welfare Act regulations, fed twice daily, and fasted for up to 14 h prior to sedation. Environmental enrichment included grouping in compound, large activity, or run-through connected cages, perches, toys, food treats, and foraging activities. If a clinical abnormality was noted, standard WaNPRC procedures were followed to notify the clinical veterinary staff for evaluation. Admission as a clinical case was solely at the discretion of clinical veterinary staff. Animals were sedated by administration of ketamine HCl and/or Telazol (tiletamine and zolazepam) and supportive agents prior to all procedures. Following sedation, animals were monitored according to WaNPRC standard protocols. For minor procedures, the presence or absence of deep pain was tested by the toe-pinch reflex. The absence of response (leg flexion) to this test indicates adequate anesthesia for a given procedure. Similar parameters were used in cases of general anesthesia, including the loss of palpebral reflexes (eye blink). Analgesics were provided as prescribed by clinical veterinary staff for at least 48 h after procedures and could be extended at the discretion of the clinical veterinarian based on clinical signs. Decisions to euthanize animals were made in close consultation with veterinary staff, and were performed in accordance with guidelines as established by the American Veterinary Medical Association Panel on Euthanasia (2013). Prior to euthanasia, animals were first rendered unconscious by administration of ketamine HCl.

Cell sources, CD34$^+$ enrichment, and *in vitro* culture

NHP CD34$^+$ cells were isolated from primed bone marrow aspirates,[49] marrow from flushed long bones ("pulp"), and/or mobilized leukapheresis.[50] Bone marrow pulp was collected post mortem from heparinized animals. Long bones (femur and humerus) were cross-sectioned with a bone saw, flushed with sterile ice-cold phosphate-buffered saline (PBS) containing 0.5% BSA (Sigma), and cleared using an MH-2150 Marrow Collection System (BioAccess, Baltimore, MD). Enriched CD34$^+$ cells were cultured overnight prior to electroporation, in 10% IMDM/FBS (Gibco, Waltham, MA) or serum-free expansion medium StemSpan SFEM II (STEMCELL Technologies, Vancouver, Canada). Each medium was supplemented with 1% penicillin-streptomycin (Gibco), and 100 ng/mL each of recombinant human stem cell factor (SCF-1) (PeproTech, Rocky Hill, NJ), thrombopoietin (TPO-1) (PeproTech), and FMS-like tyrosine kinase 3 ligand (FLT-3) (PeproTech).

Mouse experiments

NSG mice were purchased from The Jackson Laboratory or bred in-house under approved protocols and in pathogen-free housing conditions. NSG neonates (1–3 days post birth) received 150 cGy of radiation, and 3–4 h later were infused with 1×10^6 unedited or edited nonhuman primate CD34$^+$ cells in 30 μL of PBS (Gibco) containing 1% heparin (Abraxis BioScience, Los Angeles, CA) by a single intrahepatic injection. Blood samples were collected biweekly starting 8 weeks post transplant by a retro-orbital puncture. White blood cells were isolated using a BD FACS Lysing Solution (BD Biosciences, Franklin Lakes, NJ) and analyzed by flow cytometry for expression of an NHP-specific lineage marker (NHP CD45, D058-1283 from BD Biosciences). Parallel samples were reserved for isolation of total genomic DNA for NGS. After 22 weeks the animals were sacrificed, and spleen tissues were harvested and dissociated using a 70-μm filter followed by isolation of total genomic DNA.

Delivery of CCR5 ZFN mRNA

mRNA encoding NHP CCR5 ZFNs (TriLink Biotechnologies, San Diego, CA)[22] was delivered to NHP CD34$^+$ HSPCs via electroporation using either the BTX AgilePulse MAX or ECM830 (Harvard Apparatus, Holliston, MA). For experiments with the AgilePulse

MAX, 10 million $CD34^+$ HSPCs were washed thrice in Cytoporation Media T and resuspended in 1 mL of Cytoporation Media T. ZFN mRNA was added to cells at 250 μg/mL, and electroporation was conducted as previously described.[22] For experiments with the BTX ECM830, 3 million $CD34^+$ HSPCs were washed thrice with PBS (Gibco) and resuspended in 100 μL of BTXpress electroporation buffer. In an attempt to offset mRNA instability, higher concentrations of ZFN mRNA (up to 400–625 μg/mL) were utilized, followed by electroporation in 2-mm gap, 400-μL cuvettes using a single 250-V pulse for a duration of 5 ms. To extract cells from cuvettes 400 μL of fresh medium was added, and a transfer pipette was used to move cells and medium to culture dishes for recovery overnight in a 30°C, 5% CO_2 incubator. Cells were harvested 1, 2, and 5 days post electroporation for analysis of gene editing.

Delivery of CRISPR/Cas9 RNP

Chemically modified sgRNAs targeting the NHP CCR5 locus with 2′-*O*-methyl and 3′-phosphorothioate modifications at the first three 5′- and 3′-terminal RNA residues were custom-ordered from Synthego (Redwood City, CA). Lyophilized sgRNAs were resuspended in nuclease-free water at a concentration of 100 pmol/μL and stored as frozen aliquots at −80°C. TrueCut Cas9 Protein v2 (5 μg/μL) was obtained from Thermo Fisher Scientific (Waltham, MA). Prior to editing, $CD34^+$ cells were cultured for 24 h in serum-free SFEM II medium (STEMCELL) as described above. CRISPR/Cas9 RNP complexes were formed by mixing 180 pmol of Cas9 protein with 540 pmol of sgRNA and incubated at room temperature for 10 min. RNP complexes were added to 3 million $CD34^+$ HSPCs, and electroporation was conducted using the BTX ECM830 as described above. Cells were plated in fresh medium and recovered overnight in a 37°C, 5% CO_2 incubator, followed by harvest 1, 2, and 5 days post electroporation for analysis of gene editing.

RNase inhibitor assays

$CD34^+$ cells were electroporated with mRNA encoding GFP or CCR5 ZFN using the BTX ECM830 as described above, with or without RNase inhibitor (Recombinant RNasin Ribonuclease Inhibitor; Promega, Madison, WI) and recovered overnight in a 30°C, 5% CO_2 incubator. GFP expression was analyzed after 24 h by flow cytometry using the BD FACSymphony (BD Biosciences, San José, CA). ZFN mRNA expression was analyzed after 24 h by anti-Fok1 western blotting as described previously.[22,51] ZFN-based gene editing was analyzed 1, 2, and 5 days post electroporation.

Flow cytometry and sorting

Bulk $CD34^+$ cells were resuspended in FACS buffer containing sterile Dulbecco's PBS (D-PBS) (Gibco) and 1% FBS (Atlas Biologicals, Fort Collins, CO), and incubated for 15 min at 4°C with the following antibodies: anti-NHP V450 CD45 (BD Biosciences, clone D058-1283), anti-human CD34 PE-CF594 (BD Biosciences, clone 563), anti-human CD90 PE (BioLegend, clone 5E10), and anti-human CD45RA APC-Cy7 (BD Biosciences, clone 5H9). Cells were sorted for HSPC subsets on a BD FACSAria II (BD Biosciences, San José, CA) into D-PBS, followed by total genomic DNA extraction and NGS.

Colony-forming assays

ZFN- or CRISPR/Cas9-treated NHP cells (1.2×10^3) were plated in 3.6 mL of ColonyGEL 1402 (ReachBio, Seattle, WA). For colony-forming assays utilizing bone marrow cells from xenotransplanted NSG mice, cells were plated in Methocult H4434 (STEMCELL). Hematopoietic colony-forming units (CFUs) were scored after 12–14 days, based on defined morphological characteristics: CFU granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM), CFU granulocyte/macrophage (CFU-GM), CFU macrophage (CFU-M), CFU granulocyte (CFU-G), or burst-forming unit-erythrocyte (BFU-E). For measurement of biallelic CCR5 disruption in NGS assays, single colonies were collected in QuickExtract DNA Extraction Solution (Lucigen, Middleton, WI), and DNA was extracted as per manufacturer's instructions.

Next-generation sequencing

Total genomic DNA from cultured HSPCs and from hemolyzed peripheral blood and bone marrow was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) or MasterPure Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI). Genomic DNA from colony-forming assays was extracted as described above. The percentage of insertions and deletions at the CCR5 locus from each sample was quantified using a two-stage amplification protocol with Platinum Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific). The first PCR was performed using adapter primers (Table S1) spanning the CCR5 target site, followed by complete sequencing using a barcoded, 2 × 150 base-pair, paired-end MiSeq protocol, as described by the manufacturer (Illumina, San Diego, CA). Bioinformatics analysis of the sequencing data was performed using an in-house pipeline. First, paired-end reads were merged using PEAR (Paired-End reAd mergeR) with default settings.[52] A custom Python script was used for the bioinformatics analysis of the sequence data. Reads were filtered if they had more than two bases with low-quality scores before alignment. Merged reads were then aligned to the start primer and end primer sequences, allowing for two mismatches and no insertions and deletions (indels). Aligned reads were mapped to the wild-type locus sequence by Needle, a Needleman-Wunsch aligner from the EMBOSS Suite, with a gap open penalty of 10.0 and gap extend penalty of 0.5 (http://emboss.sourceforge.net/apps/release/6.6/emboss/apps/needle.html). Reads were grouped based on the alignment pattern as (1) reads that match the wild-type sequence, (2) reads with substitutions only, and (3) reads with distinct indel patterns. A custom R script was used to generate bar graphs to show the editing frequency of the samples using the ggplot2 package.[53]

Statistics

All statistical analyses were performed using GraphPad Prism 7.03 software utilizing unpaired two-tailed t tests or nonparametric Mann-Whitney tests (not significant, $p > 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as shown in figures).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.11.010.

ACKNOWLEDGMENTS

The authors thank Helen Crawford for assistance in preparing the manuscript, and Veronica Nelson, Erica Wilson, Kelvin Sze, Megan Brown, Sarah Herrin, Alan Ung, and Michelle Hoffman for outstanding support in our NHP studies. We are grateful to Christina Ironside and Kevin Haworth for the supporting mouse xenograft studies, Lucrezia Colonna and Leslie Kean for provision of cryopreserved rhesus $CD34^+$ cells and methods for postmortem bone marrow collection, and Teresa Einhaus for feedback on western blot assays. This study was supported by grants from the National Institutes of Health, National Heart, Lung, and Blood Institute (U19 HL129902 and U19 HL156247 to H.-P.K.), and National Institute of Allergy and Infectious Diseases (UM1 AI126623, U19 AI149676, U19 AI149680, R01 AI135953, and R01 HL136135 to H.-P.K.). All primate work was completed at the WaNPRC, which is supported by U42 (U42 OD011123) and P51 (P51 OD010425) grants through the NIH Office of Research Infrastructure Programs. All data supporting the findings of this study are available within the paper and its supplementary information files, or from the corresponding authors upon request.

AUTHOR CONTRIBUTIONS

H.-P.K. and C.W.P. are the principal investigators of the study and designed and coordinated the overall execution of the project. C.W.P. and O.H. designed the animal experiments. S.S.R., S.R., and C.W.P. performed the HSPC sorting and mouse xenograft experiments. R.V. and S.S.R. performed the RNase inhibitor assays. R.V., C.W.P., and O.H. optimized CRISPR/Cas9 RNPs targeting NHP CCR5. C.W.P., R.V., and S.S.R. curated and analyzed the data. D.P. and M.R.E. performed bioinformatics analyses of NGS data. C.W.P., R.V., and S.S.R. wrote the manuscript, with feedback from all co-authors.

DECLARATION OF INTERESTS

H.P.K. has received support as the inaugural recipient of the José Carreras/E. Donnall Thomas Endowed Chair for Cancer Research and the Stephanus Family Endowed Chair for Cell and Gene Therapy, and is or was a consultant to and has or had ownership interests with Rocket Pharmaceuticals, Homology Medicines, VOR Biopharma, and Ensoma Inc. H.P.K. has also been a consultant to CSL Behring and Magenta Therapeutics. S.R. is a consultant to Forty Seven Inc. (Gilead Sciences) and Ensoma Inc.

REFERENCES

1. Sharma, G., Sharma, A.R., Bhattacharya, M., Lee, S.S., and Chakraborty, C. (2020). CRISPR-Cas9: a preclinical and clinical perspective for the treatment of human diseases. Mol. Ther *29*, 571–586.
2. Zhang, H.X., Zhang, Y., and Yin, H. (2019). Genome editing with mRNA encoding ZFN, TALEN, and Cas9. Mol. Ther. *27*, 735–746.
3. Bogdanove, A.J., Bohm, A., Miller, J.C., Morgan, R.D., and Stoddard, B.L. (2018). Engineering altered protein-DNA recognition specificity. Nucleic Acids Res. *46*, 4845–4871.
4. Mustafa, M.I., and Makhawi, A.M. (2021). SHERLOCK and DETECTR: CRISPR-Cas systems as potential rapid diagnostic tools for emerging infectious diseases. J. Clin. Microbiol. *59*, e00745-20.
5. Fozouni, P., Son, S., Diaz de Leon Derby, M., Knott, G.J., Gray, C.N., D'Ambrosio, M.V., Zhao, C., Switz, N.A., Kumar, G.R., Stephens, S.I., et al. (2021). Amplification-free detection of SARS-CoV-2 with CRISPR-Cas13a and mobile phone microscopy. Cell *184*, 323–333 e329.
6. Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J., et al. (2011). A TALE nuclease architecture for efficient genome editing. Nat. Biotechnol. *29*, 143–148.
7. Urnov, F.D., Miller, J.C., Lee, Y.L., Beausejour, C.M., Rock, J.M., Augustus, S., Jamieson, A.C., Porteus, M.H., Gregory, P.D., and Holmes, M.C. (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature *435*, 646–651.
8. Wiedenheft, B., Sternberg, S.H., and Doudna, J.A. (2012). RNA-guided genetic silencing systems in bacteria and archaea. Nature *482*, 331–338.
9. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823–826.
10. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science *339*, 819–823.
11. Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., and Liu, D.R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature *533*, 420–424.
12. Kim, Y.B., Komor, A.C., Levy, J.M., Packer, M.S., Zhao, K.T., and Liu, D.R. (2017). Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. Nat. Biotechnol. *35*, 371–376.
13. Wienert, B., Wyman, S.K., Richardson, C.D., Yeh, C.D., Akcakaya, P., Porritt, M.J., Morlock, M., Vu, J.T., Kazane, K.R., Watry, H.L., et al. (2019). Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. Science *364*, 286–289.
14. Tsai, S.Q., Nguyen, N.T., Malagon-Lopez, J., Topkar, V.V., Aryee, M.J., and Joung, J.K. (2017). CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. Nat. Methods *14*, 607–614.
15. Kim, D.Y., Moon, S.B., Ko, J.H., Kim, Y.S., and Kim, D. (2020). Unbiased investigation of specificities of prime editing systems in human cells. Nucleic Acids Res. *48*, 10576–10589.
16. Stadtmauer, E.A., Fraietta, J.A., Davis, M.M., Cohen, A.D., Weber, K.L., Lancaster, E., Mangan, P.A., Kulikovskaya, I., Gupta, M., Chen, F., et al. (2020). CRISPR-engineered T cells in patients with refractory cancer. Science *367*, eaba7365.
17. Charlesworth, C.T., Deshpande, P.S., Dever, D.P., Camarena, J., Lemgart, V.T., Cromer, M.K., Vakulskas, C.A., Collingwood, M.A., Zhang, L., Bode, N.M., et al. (2019). Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nat. Med. *25*, 249–254.
18. Li, A., Tanner, M.R., Lee, C.M., Hurley, A.E., De Giorgi, M., Jarrett, K.E., Davis, T.H., Doerfler, A.M., Bao, G., Beeton, C., et al. (2020). AAV-CRISPR gene editing is negated by pre-existing immunity to Cas9. Mol. Ther. *28*, 1432–1441.
19. Diener, Y., Bosio, A., and Bissels, U. (2016). Delivery of RNA-based molecules to human hematopoietic stem and progenitor cells for modulation of gene expression. Exp. Hematol. *44*, 991–1001.
20. Peterson, C.W., and Kiem, H.P. (2019). Lessons from London and Berlin: designing a scalable gene therapy approach for HIV cure. Cell Stem Cell *24*, 685–687.
21. Gomez-Ospina, N., Scharenberg, S.G., Mostrel, N., Bak, R.O., Mantri, S., Quadros, R.M., Gurumurthy, C.B., Lee, C., Bao, G., Suarez, C.J., et al. (2019). Human genome-edited hematopoietic stem cells phenotypically correct mucopolysaccharidosis type I. Nat. Commun. *10*, 4045.
22. Peterson, C.W., Wang, J., Norman, K.K., Norgaard, Z.K., Humbert, O., Tse, C.K., Yan, J.J., Trimble, R.G., Shivak, D.A., Rebar, E.J., et al. (2016). Long-term multilineage engraftment of autologous genome-edited hematopoietic stem cells in nonhuman primates. Blood *127*, 2416–2426.
23. Humbert, O., Peterson, C.W., Norgaard, Z.K., Radtke, S., and Kiem, H.P. (2018). A nonhuman primate transplantation model to evaluate hematopoietic stem cell gene editing strategies for beta-hemoglobinopathies. Mol. Ther. Methods Clin. Dev. *8*, 75–86.

24. Peterson, C.W., Wang, J., Deleage, C., Reddy, S., Kaur, J., Polacino, P., Reik, A., Huang, M.L., Jerome, K.R., Hu, S.L., et al. (2018). Differential impact of transplantation on peripheral and tissue-associated viral reservoirs: implications for HIV gene therapy. PLoS Pathog. *14*, e1006956.

25. Paul, B., Ibarra, G.S.R., Hubbard, N., Einhaus, T., Astrakhan, A., Rawlings, D.J., Kiem, H.P., and Peterson, C.W. (2018). Efficient enrichment of gene-modified primary T cells via CCR5-targeted integration of mutant dihydrofolate reductase. Mol. Ther. Methods Clin. Dev. *9*, 347–357.

26. Humbert, O., Radtke, S., Samuelson, C., Carrillo, R.R., Perez, A.M., Reddy, S.S., Lux, C., Pattabhi, S., Schefter, L.E., Negre, O., et al. (2019). Therapeutically relevant engraftment of a CRISPR-Cas9-edited HSC-enriched population with HbF reactivation in nonhuman primates. Sci. Transl. Med. *11*, eaaw3768.

27. Rust, B.J., Kean, L.S., Colonna, L., Brandenstein, K.E., Poole, N.H., Obenza, W., Enstrom, M.R., Maldini, C.R., Ellis, G.I., Fennessey, C.M., et al. (2020). Robust expansion of HIV CAR T cells following antigen boosting in ART-suppressed nonhuman primates. Blood *136*, 1722–1734.

28. DiGiusto, D.L., Cannon, P.M., Holmes, M.C., Li, L., Rao, A., Wang, J., Lee, G., Gregory, P.D., Kim, K.A., Hayward, S.B., et al. (2016). Preclinical development and qualification of ZFN-mediated CCR5 disruption in human hematopoietic stem/progenitor cells. Mol. Ther. Methods Clin. Dev. *3*, 16067.

29. Wang, J., Exline, C.M., DeClercq, J.J., Llewellyn, G.N., Hayward, S.B., Li, P.W., Shivak, D.A., Surosky, R.T., Gregory, P.D., Holmes, M.C., et al. (2015). Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors. Nat. Biotechnol. *33*, 1256–1263.

30. Holt, N., Wang, J., Kim, K., Friedman, G., Wang, X., Taupin, V., Crooks, G.M., Kohn, D.B., Gregory, P.D., Holmes, M.C., et al. (2010). Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. Nat. Biotechnol. *28*, 839–847.

31. Cardozo-Ojeda, E.F., Duke, E.R., Peterson, C.W., Reeves, D.B., Mayer, B.T., Kiem, H.P., and Schiffer, J.T. (2021). Thresholds for post-rebound SHIV control after CCR5 gene-edited autologous hematopoietic cell transplantation. eLife *10*, e57646. https://doi.org/10.7554/eLife.57646.

32. Hutter, G., Nowak, D., Mossner, M., Ganepola, S., Mussig, A., Allers, K., Schneider, T., Hofmann, J., Kucherer, C., Blau, O., et al. (2009). Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. N. Engl. J. Med. *360*, 692–698.

33. Gupta, R.K., Abdul-Jawad, S., McCoy, L.E., Mok, H.P., Peppa, D., Salgado, M., Martinez-Picado, J., Nijhuis, M., Wensing, A.M.J., Lee, H., et al. (2019). HIV-1 remission following CCR5Delta32/Delta32 haematopoietic stem-cell transplantation. Nature *568*, 244–248.

34. Radtke, S., Adair, J.E., Giese, M.A., Chan, Y.Y., Norgaard, Z.K., Enstrom, M., Haworth, K.G., Schefter, L.E., and Kiem, H.P. (2017). A distinct hematopoietic stem cell population for rapid multilineage engraftment in nonhuman primates. Sci. Transl. Med. *9*, eaan1145.

35. Horn, P.A., Thomasson, B.M., Wood, B.L., Andrews, R.G., Morris, J.C., and Kiem, H.P. (2003). Distinct hematopoietic stem/progenitor cell populations are responsible for repopulating NOD/SCID mice compared with nonhuman primates. Blood *102*, 4329–4335.

36. Frangoul, H., Altshuler, D., Cappellini, M.D., Chen, Y.S., Domm, J., Eustace, B.K., Foell, J., de la Fuente, J., Grupp, S., Handgretinger, R., et al. (2021). CRISPR-Cas9 gene editing for sickle cell disease and beta-thalassemia. N. Engl. J. Med. *384*, 252–260.

37. McCaffrey, A.P., Ohashi, K., Meuse, L., Shen, S., Lancaster, A.M., Lukavsky, P.J., Sarnow, P., and Kay, M.A. (2002). Determinants of hepatitis C translational initiation in vitro, in cultured cells and mice. Mol. Ther. *5*, 676–684.

38. Dever, D.P., Bak, R.O., Reinisch, A., Camarena, J., Washington, G., Nicolas, C.E., Pavel-Dinu, M., Saxena, N., Wilkens, A.B., Mantri, S., et al. (2016). CRISPR/Cas9 beta-globin gene targeting in human haematopoietic stem cells. Nature *539*, 384–389.

39. Hendel, A., Bak, R.O., Clark, J.T., Kennedy, A.B., Ryan, D.E., Roy, S., Steinfeld, I., Lunstad, B.D., Kaiser, R.J., Wilkens, A.B., et al. (2015). Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat. Biotechnol. *33*, 985–989.

40. Liang, X., Potter, J., Kumar, S., Zou, Y., Quintanilla, R., Sridharan, M., Carte, J., Chen, W., Roark, N., Ranganathan, S., et al. (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. J. Biotechnol. *208*, 44–53.

41. Vaidyanathan, S., Azizian, K.T., Haque, A., Henderson, J.M., Hendel, A., Shore, S., Antony, J.S., Hogrefe, R.I., Kormann, M.S.D., Porteus, M.H., et al. (2018). Uridine depletion and chemical modification increase Cas9 mRNA activity and reduce immunogenicity without HPLC purification. Mol. Ther. Nucleic Acids *12*, 530–542.

42. Jiang, T., Henderson, J.M., Coote, K., Cheng, Y., Valley, H.C., Zhang, X.O., Wang, Q., Rhym, L.H., Cao, Y., Newby, G.A., et al. (2020). Chemical modifications of adenine base editor mRNA and guide RNA expand its application scope. Nat. Commun. *11*, 1979.

43. Rees, H.A., and Liu, D.R. (2018). Base editing: precision chemistry on the genome and transcriptome of living cells. Nat. Rev. Genet. *19*, 770–788.

44. Newby, G.A., Yen, J.S., Woodard, K.J., Mayuranathan, T., Lazzarotto, C.R., Li, Y., Sheppard-Tillman, H., Porter, S.N., Yao, Y., Mayberry, K., et al. (2021). Base editing of haematopoietic stem cells rescues sickle cell disease in mice. Nature *595*, 295–302.

45. Devoldere, J., Dewitte, H., De Smedt, S.C., and Remaut, K. (2016). Evading innate immunity in nonviral mRNA delivery: don't shoot the messenger. Drug Discov. Today *21*, 11–25.

46. Guan, S., and Rosenecker, J. (2017). Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems. Gene Ther. *24*, 133–143.

47. Kariko, K., Muramatsu, H., Ludwig, J., and Weissman, D. (2011). Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res. *39*, e142.

48. Anderson, B.R., Muramatsu, H., Jha, B.K., Silverman, R.H., Weissman, D., and Kariko, K. (2011). Nucleoside modifications in RNA limit activation of 2′-5′-oligoadenylate synthetase and increase resistance to cleavage by RNase L. Nucleic Acids Res. *39*, 9329–9338.

49. Radtke, S., Perez, A.M., Venkataraman, R., Reddy, S., Haworth, K.G., Humbert, O., Kiem, H.P., and Peterson, C.W. (2019). Preparation and gene modification of nonhuman primate hematopoietic stem and progenitor cells. J. Vis. Exp, e58933. https://doi.org/10.3791/58933.

50. Wu, H.L., Greene, J.M., Swanson, T., Shriver-Munsch, C., Armantrout, K., Weber, W.C., Bateman, K.B., Maier, N.M., Northrup, M., Legasse, A.W., et al. (2020). Terumo spectra optia leukapheresis of cynomolgus macaques for hematopoietic stem cell and T cell collection. J. Clin. Apher. *36*, 67–77.

51. Stoltzman, C.A., Peterson, C.W., Breen, K.T., Muoio, D.M., Billin, A.N., and Ayer, D.E. (2008). Glucose sensing by MondoA:Mlx complexes: a role for hexokinases and direct regulation of thioredoxin-interacting protein expression. Proc. Natl. Acad. Sci. U S A *105*, 6912–6917.

52. Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics *30*, 614–620.

53. Wickham, H. (2016). ggplot2. Elegant Graphics for Data Analysis (Springer International Publishing).