Received: 22 January 2024 | Revised: 22 April 2024 | Accepted: 22 April 2024

https://doi.org/10.1016/j.jtha.2024.04.020

ORIGINAL ARTICLE

DNA base editing corrects common hemophilia A mutations and restores factor VIII expression in *in vitro* and *ex vivo* models

Elena Tonetto[1] | Alessia Cucci[2] | Antonia Follenzi[2] | Francesco Bernardi[1] | Mirko Pinotti[1] | Dario Balestra[1]

[1]Department of Life Sciences and Biotechnology and Laboratorio per le Tecnologie delle Terapie Avanzate (LTTA), University of Ferrara, Ferrara, Italy

[2]Department of Health Sciences, School of Medicine, University of Piemonte Orientale, Novara, Italy

Correspondence
Mirko Pinotti, Department of Life Sciences and Biotechnology, University of Ferrara, Via Fossato di Mortara 74, 44121 Ferrara, Italy.
Email: pnm@unife.it

Funding information
This work was supported by grants to M.P. from Bayer (Bayer Hemophilia Award Program BHAP 2020) and to D.B. from Novo Nordisk (Novo Nordisk Access to Insight Grants Program 2022) and Associazione Italiana Centri Emofilia (AICE). A.F. was supported by Telethon grant number GGP19201.

Abstract

Background: Replacement and nonreplacement therapies effectively control bleeding in hemophilia A (HA) but imply lifelong interventions. Authorized gene addition therapy could provide a cure but still poses questions on durability. *FVIII* gene correction would definitively restore factor (F)VIII production, as shown in animal models through nuclease-mediated homologous recombination (HR). However, low efficiency and potential off-target double-strand break still limit HR translatability.

Objectives: To correct common model single point mutations leading to severe HA through the recently developed double-strand break/HR-independent base editing (BE) and prime editing (PE) approaches.

Methods: Screening for efficacy of BE/PE systems in HEK293T cells transiently expressing FVIII variants and validation at DNA (sequencing) and protein (enzyme-linked immunosorbent assay; activated partial thromboplastin time) level in stable clones. Evaluation of rescue in engineered blood outgrowth endothelial cells by lentiviral-mediated delivery of BE.

Results: Transient assays identified the best-performing BE/PE systems for each variant, with the highest rescue of FVIII expression (up to 25% of wild-type recombinant FVIII) for the p.R2166* and p.R2228Q mutations. In stable clones, we demonstrated that the mutation reversion on DNA (∼24%) was consistent with the rescue of FVIII secretion and activity of 20% to 30%. The lentiviral-mediated delivery of the selected BE systems was attempted in engineered blood outgrowth endothelial cells harboring the p.R2166* and p.R2228Q variants, which led to an appreciable and dose-dependent rescue of secreted functional FVIII.

Conclusion: Overall data provide the first proof-of-concept for effective BE/PE-mediated correction of HA-causing mutations, which encourage studies in mouse models to develop a personalized cure for large cohorts of patients through a single intervention.

Manuscript handled by: Ton Lisman

Final decision: Ton Lisman, 22 April 2024

KEYWORDS
base/prime editors, CRISPR, factor VIII, gene editing, hemophilia A

1 | INTRODUCTION

Hemophilia A (HA; OMIM #306700) is a rare X-linked recessive disorder caused by mutations in the *FVIII* gene (MIM 300841), which encodes coagulation factor (F)VIII [1]. FVIII is physiologically produced mainly by liver sinusoidal endothelial cells, and it has also been demonstrated in extra-hepatic production with organ-specific endothelial cells and hematopoietic cells [2–4].

Current treatment of severe HA is based on lifelong prophylactic injection of FVIII (replacement therapy), which however suffers from a short half-life and imposes frequent administrations. This limitation, combined with the development of anti-FVIII neutralizing antibodies in ~30% of treated HA patients [5], boosted research and led to several alternative strategies ranging from the use of extended-half-life FVIII variants to bypassing agents and inhibitors of the physiologic coagulation inhibitors, some of them also into the clinics [6,7]. On the other hand, to provide a definitive cure for HA, strong efforts have been pushed on gene therapy [8], and very recently, valoctocogene roxaparvovec [9], an adeno-associated viral vector delivering the B-domain deleted (BDD) FVIII variant, has received authorization by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA). Clinical trials [9–13] demonstrated an appreciable increase of FVIII activity in plasma over time after a single treatment and a subsequent remarkable reduction of bleeding episodes, allowing most treated HA patients to discontinue prophylaxis. Notwithstanding, the follow-up revealed a decline in the FVIII activity levels over the years, thus posing the question on the durability of this therapeutic effect. Moreover, this approach relies on the episomal persistence of the therapeutic transgene in hepatocytes, which inherently restricts its application to adults only [14,15].

In this context, genome editing could provide a definitive cure with a single intervention, as it has been elegantly shown in mouse models for HA via homologous recombination (HR) triggered by nuclease-driven double-strand break (DSB) [16–20]. Notwithstanding, new DSB/HR-independent DNA editing approaches stemming from the CRISPR-Cas technology have been recently developed (Figure 1), thus opening new therapeutic perspectives for human genetic disorders.

Base editors (BEs) comprise a catalytically impaired Cas9 nickase delivered on target by its specific guide RNA (gRNA) and a base modification enzyme able to edit a single nucleotide on single-stranded nucleic acid substrate [21–25]. Cytosine BEs, able to convert C into T, and adenine base editors (ABEs), able to convert A into G, can collectively mediate all 4 possible transition mutations (C > T, A > G, or in the opposite strand, T > C and G > A). These BEs have been delivered to a wide range of tissue targets with viral and nonviral modalities to treat genetic disorders [26–30], and some are currently in clinical trials [31,32].

On the other hand, prime editors (PEs) can mediate targeted insertions, deletions, all 12 possible base-to-base conversions, and combinations thereof [33]. The PE2 architecture is based on a Cas9 nickase fused to an engineered reverse transcriptase (RT) domain that is targeted to the editing site by an engineered prime editing guide RNA (pegRNA), which specifies the target site in its spacer sequence (gRNA). The pegRNA also contains a primer binding sequence and an RT template sequence encoding the desired edit. Once landed on the target site, the PE cuts the opposite strand, and the flapping DNA is hybridized with the primer binding sequence, thus providing the 3′ end exploited by the RT to synthesize new DNA that is then inserted into the genome. To further promote the incorporation of the newly synthesized DNA, a simple single guide RNA (sgRNA) can be added to direct PE2 to nick the nonedited strand, thus forming the PE3/PE3b system. Optimization of the PE2 and PE3 architectures and incorporation of proteins to manipulate DNA repair machinery led to the development of the recent and more efficient PE4, PE5, and PE6 variants [34,35]. Since its discovery, PE has been applied to a broad type of targets, ranging from cultured cells to human pluripotent stem cells, as well as disease mouse models, either to model or to rescue pathogenic mutations [33–36].

Here, for the first time, we applied the BE and PE approaches to HA-causing point mutations. Collectively, more than 3000 unique variants have been reported in the European Association for Haemophilia and Allied Disorders and the Human Gene Mutation databases (European Association for Haemophilia and Allied Disorders: http://f8-db.eahad.org/; Human Gene Mutation: https://www.hgmd.cf.ac.uk/) [37]. Among them, besides the large InterVening Sequence (IVS) 1 and IVS22 inversions [38,39] that are not approachable with the proposed BE/PE system, point mutations (missense, nonsense, and splicing variants) were found in over 30% of severe HA patients and in the vast majority of moderate forms.

Through investigations on a panel of highly recurrent and representative missense/nonsense model mutations associated with severe HA, we demonstrated in cellular models that BE and PE systems can be tailored to efficiently correct point mutations at the DNA level, ultimately resulting in a consistent rescue of recombinant FVIII (rFVIII) secretion and function.

2 | METHODS

2.1 | Bioinformatics analysis

The selection and design of gRNA and pegRNA were performed by exploiting the following bioinformatic tools: CRISPR RGEN Tools [40,41], pegIT [42], pegFinder [43], BE-Designer [44], BE-HIVE [45], and CRISPOR [46].

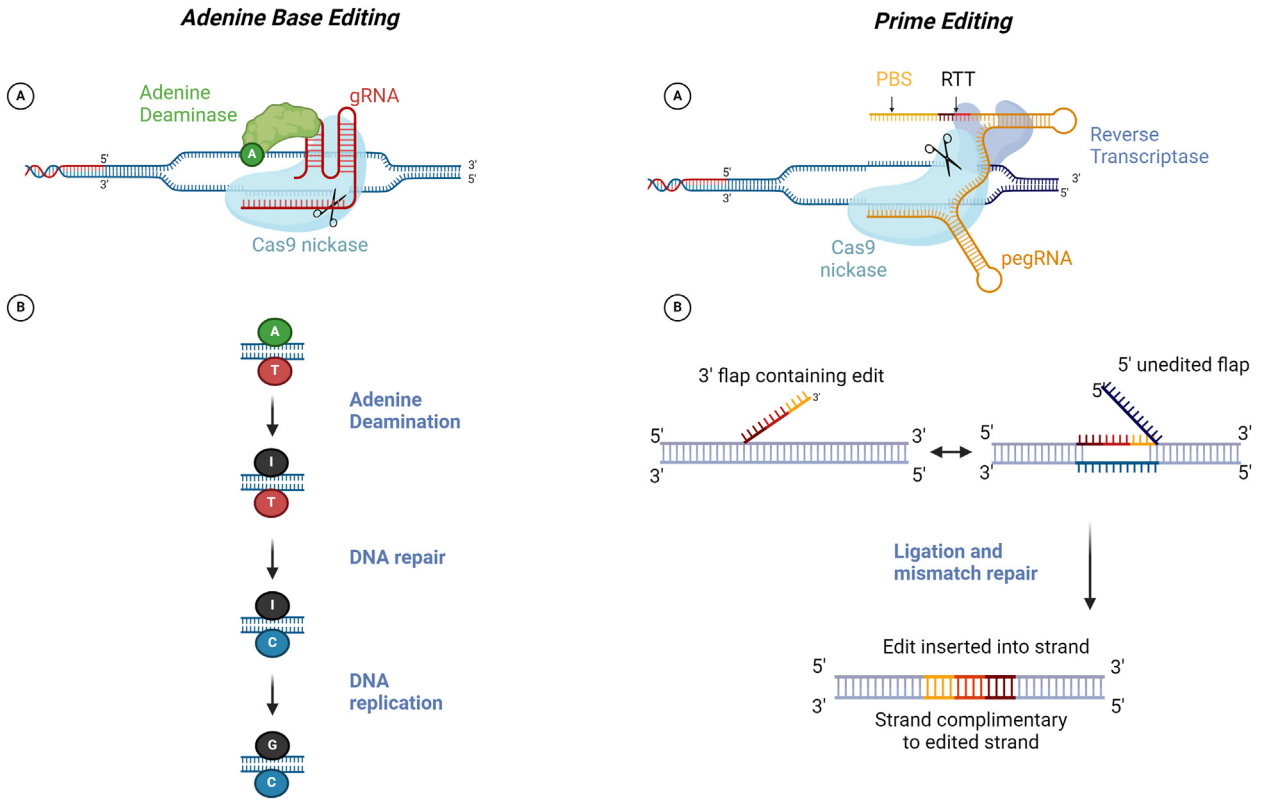


FIGURE 1 The adenine base editing (ABE) and prime editing (PE) tools. Schematic representation of the ABE (left) and PE (right) tools. ABE is composed of a Cas9 nickase targeting a specific DNA sequence through a specific guide RNA (gRNA). A directly evolved adenine deaminase, fused to Cas9 nickase, is responsible for deaminating the target adenine (A) to inosine (I). Cellular DNA repair machinery recognizes the mismatch (I:T) and repairs the opposite nicked strand using the inosine (I) as a template. After DNA replication, the repair process results in an A-to-G conversion at the target site. PE is composed of a reverse transcriptase (RT) fused to a Cas9 nickase, targeted to a specific DNA sequence through a specific prime editing guide RNA (pegRNA). The pegRNA also provides a primer binding site (PBS) that base pairs with the cut DNA strand and is exploited by the RT to synthesize new DNA by copying the information by the RT template (RTT), also coded by the pegRNA. The newly synthesized complementary DNA (cDNA) strand displaces a short segment of the target DNA strand (flap) due to complementarity with the target sequence and the RTT. Cellular DNA repair machinery recognizes the nick and displaced flap. The flap is removed, and the edited cDNA strand is integrated into the target DNA using the opposite strand as a template.

To quantify editing at DNA level, Sanger sequencing results were analyzed with the EditR tool [47].

2.2 | Construction of expression plasmids

The generation of expression plasmids for the rFVIII variants was outsourced to Twist Bioscience Company. Briefly, the lentiviral pF8 BDD plasmid expressing the BDD FVIII isoform was edited by inserting a synthesized cassette containing the internal ribosome entry site-PuroR fragments into the *XbaI* and *SalI* restriction sites. Then, the FVIII portion between the *BsiWI* and *XbaI* restriction sites was replaced by a synthesized fragment containing the selected FVIII variants. All constructs were validated by next-generation sequencing.

The pU6-pegRNA-GG-acceptor and MLM3636 plasmids were purchased from AddGene (plasmids #132777 and #43860). Cloning in these plasmids was done as described by Anzalone et al. [33]. Oligonucleotides used for the construction of pegRNAs and gRNAs were purchased from Integrated DNA Technology and listed in Supplementary Table S1.

Plasmids are listed in Supplementary Table S2.

2.3 | Transient transfection assays, generation of rFVIII stable clones, and editing

Human embryonic kidney 293T (HEK293T, ATCC CRL-3216) cells were transiently transfected in 24-well plates with plasmid pFVIII vectors (800 ng) without or with plasmids for the combination of BE/PE (650 ng) and gRNA/pegRNA (50 ng) with Lipofectamine 2000 (Invitrogen), as per manufacturer's instructions. The culture medium was replaced 4 hours posttransfection with fresh OptiMEM (Sigma-Aldrich). Media were harvested 48 hours after transfection, centrifuged for 5 minutes at 3000 × *g*, and stored at −20 °C.

To generate rFVIII-expressing cell lines, HEK293T cells were transfected with the *ScaI*-linearized rFVIII-expressing plasmids in 24-

well plates with Lipofectamine 2000. After 3 days of recovery, cells were selected with 1 μg/mL Puromycin (Sigma-Aldrich). A stable pool of clonal cell lines was isolated, and plasmid integration was confirmed by polymerase chain reaction (PCR; data not shown). To assess editing, stable clones were transfected with a combination of BE/PE (1.2 μg) and gRNA/pegRNA (110 ng) as reported above.

2.4 | Lentiviral vector production

The lentiviral pF8 BDD plasmids used to transiently express rFVIII were used to generate rFVIII-lentiviral vectors (LVs). The recombinant lentiviral plasmids expressing the ABE8e-NG together with its mutation-specific gRNA were generated by exploiting the golden-gate protocol (NEBridge Golden Gate Assembly; New England Biolabs). Briefly, fragments containing the lentiviral backbone (region spanning the internal ribosome entry site through central polypurine tract/central termination sequence - cPPT/CTS), ABE8e-NG, and gRNA expression cassettes were PCR amplified from plasmids SIN40C.SFFV.MCS.IRES.GFP, NG-ABE8e, and MLM3636 (AddGene plasmids #169280, #138491, and #43860, respectively) with high-fidelity Q5 polymerase and joined by exploiting the *BsmBI*-v2 type IIS restriction site. Primers and 4-base overhangs were designed by exploiting the NEBridge Golden Gate Assembly Tool (New England Biolabs).

LVs were produced by transient transfection in HEK293T cells using the calcium phosphate method and pMDLg/pRRE, pVSV-G, and pRSV-Rev (Addgene #12251, #138479, and #12253, respectively) as packaging plasmids. LVs were harvested at 48 hours posttransfection and filtered through 0.22-μm pore cellulose acetate filters. LVs were concentrated by ultracentrifugation (2 hours at 50 000 × *g* at room temperature). LV titers were calculated by green fluorescent protein analysis of HEK293T transduced by serial dilution of LVs.

2.5 | Generation of engineered blood outgrowth endothelial cells and editing

Blood outgrowth endothelial cells (BOECs) from a severe HA patient harboring the *FVIII* IVS22 inversion [48] were plated at a 10^4 cells/cm^2 density and transduced with LVs carrying the wild-type (WT) FVIII (FVIIIwt) or the FVIII R2166* or R2228Q variants (LV-FVIIIwt, LV-FVIII-R2166*, LV-FVIII-R2228Q, respectively) at multiplicity of infection (MOI) of 10. After 72 hours, cells were selected with 1 μg/mL of Puromycin (Sigma-Aldrich) for 3 days. The number of integrated copies of each construct was comparable, more specifically, 5, 6, and 4, respectively. HA BOECs were subsequentially transduced with LV expressing the ABE8e-NG and the mutation-specific gRNAs at MOI of 1.25, 2.5, 5, 10, and 20. LV-transduced HA BOECs were cultured for 72 hours, and the supernatant was collected and stored at −20 °C.

2.6 | Measurement of rFVIII protein levels

Secreted rFVIII levels and cofactor FVIII activity were determined in cell media through a commercial enzyme-linked immunosorbent assay kit (F8C-EIA, Affinity Biologicals) and activated partial thromboplastin time (APTT) assay with FVIII depleted plasma (Hyphen Biomed), respectively. Secreted rFVIII antigen and activity levels were expressed as percent (%) of the rFVIIIwt, and specific activity was calculated as the ratio between activity and protein levels. All experiments were run in duplicate and repeated 3 times.

2.7 | Evaluation of correction at DNA level

Genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Promega). PCR reactions were carried out with 400 ng of genomic DNA and plasmid-specific primers. The primers used are listed in Supplementary Table S1. PCR products were purified with the QIAquick Gel Extraction kit (QIAGEN) and subjected to Sanger sequencing. Results were analyzed by EditR tool.

2.8 | Statistical analysis

Data analysis was performed by unpaired *t*-test with Welch's correction. A value of $P < .05$ was considered statistically significant for a 95% CI.

2.9 | Data sharing statement

Relevant data are included in the article and can be found in Supplementary Data available with the online version of this article. For original data, please contact pnm@unife.it or blsdra@unife.it. Reagents are available under material transfer agreement on request from the corresponding author, Mirko Pinotti (pnm@unife.it).

3 | RESULTS

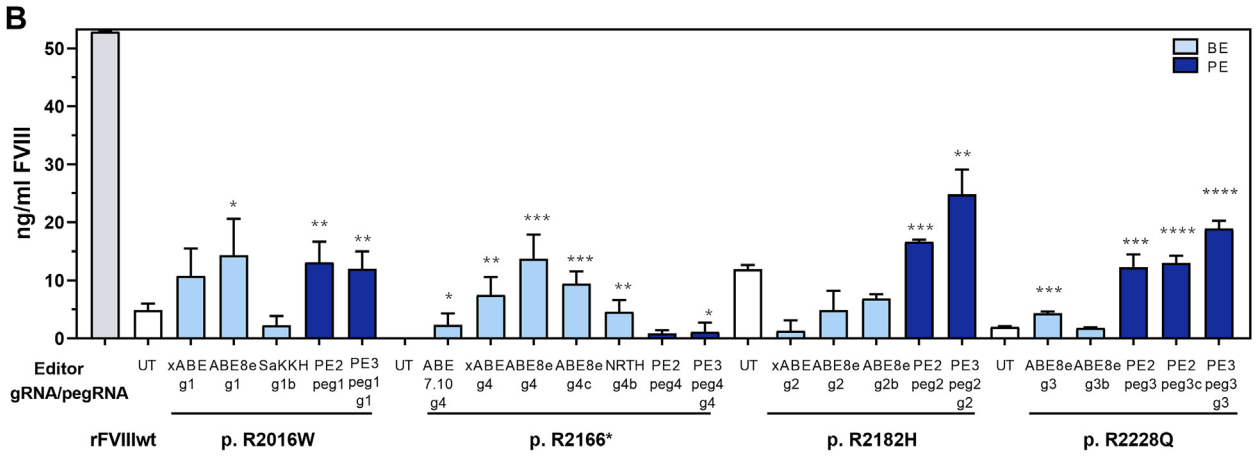
Among all *FVIII* point mutations having sequence requirements amenable for the correction through the BE and PE systems (Figure 1), we selected 4 common *FVIII* missense and nonsense variants associated with moderate-to-severe bleeding phenotypes (Figure 2A).

3.1 | Characterization of rFVIII variants and selection of active BEs and PEs

To build an experimental model to screen BE/PE for efficiency, we transiently expressed the native BDD FVIII variants in HEK293T cells. As shown in Figure 2B, all mutations led to remarkably reduced FVIII

A

Mutation type	Location	No of Patients	Nucleotide change	Protein change	FVIII:C%	FVIII:Ag%
Missense	Exon 19	100	c.6046C>T	p.R2016W	1	3,5
Nonsense	Exon 23	54	c.6496C>T	p.R2166*	<1	<1
Missense	Exon 23	67	c.6545G>A	p.R2182H	<1	n.r
Missense	Exon 24	57	c.6683G>A	p.R2228Q	<1	1



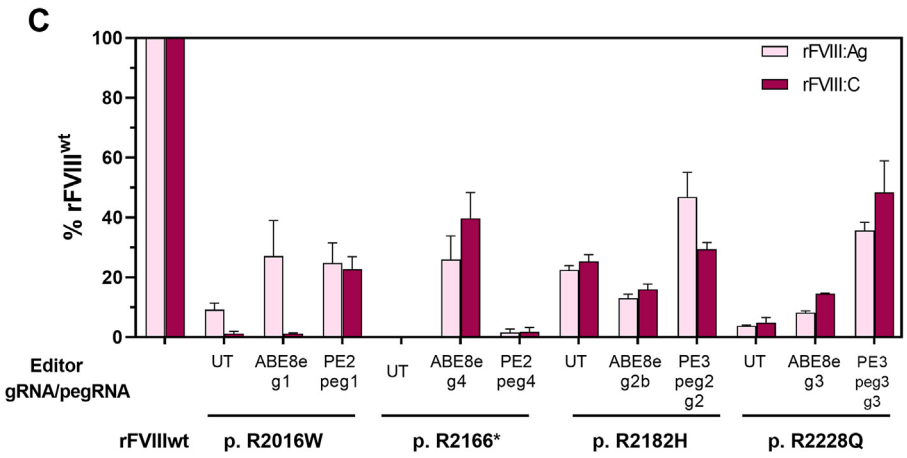


FIGURE 2 Base Editor (BE)/Prime Editor (PE) can rescue recombinant factor VIII (rFVIII) secretion and coagulant activity impaired by common Hemophilia A point mutations. (A) Features of the 4 representative *FVIII* missense/nonsense mutations selected for this study. Data come from the FVIII Gene (FVIII) Variant Database (European Association for Haemophilia and Allied Disorders; accessed on January 6, 2020). Median of FVIII activity (FVIII:C) and antigen (FVIII:Ag) levels are reported. (B) FVIII:Ag levels in media from HEK293T cells transiently transfected with rFVIII expression plasmids alone (UT, white bars) or in combination with BE (light blue bars) or PE (blue bars). Results are expressed as ng/mL. Results are presented as mean ± SD of 3 independent experiments. *$P < .1$; **$P < .01$; ***$P < .001$; ****$P < .0001$. (C) FVIII:Ag (light red) and FVIII:C (red) levels evaluated in media from HEK293T cells transiently transfected with rFVIII expression plasmids alone (UT) or in combination with BE or PE. FVIII:Ag and FVIII:C levels are reported as the percentage of wild-type rFVIII (rFVIIIwt). Results are presented as mean ± SD of 3 independent experiments. ABE, adenine base editor; gRNA, guide RNA; pegRNA, prime editing guide RNA; UT, untreated.

secreted levels compared with rFVIIIwt (52.9 ± 0.2 ng/mL), albeit to a graded extent (p.R2182H, 11.9 ± 0.8 ng/mL; p.R2016W, 4.9 ± 1.1 ng/mL; p.R2228Q, 2.0 ± 0.1 ng/mL; p.R2166*, <0.1 ng/mL). Conversely, evaluation of the coagulant properties by the APTT assay indicated that the p.R2016W led to a dysfunctional rFVIII isoform, as witnessed by the 1.2% of activity compared with rFVIIIwt, likely due to the bystander effect (Figure 2). Differently, the p.R2182H and p.R2228Q variants were associated with coherent reduction of coagulant activity (25.3% and 4.8%, respectively). As expected, we did not detect any appreciable activity in conditioned medium from the p.R2166* expressing cells compared with the negative control.

By taking advantage of bioinformatics tools, we designed for each variant a panel of gRNA and pegRNA to be combined with the xABE7.10, ABE8e-NG, ABE8e-SaKKH, ABE7.10, ABEmax-NRTH, and Sp-PE2/PE3 editors. To screen them for efficiency, cells were cotransfected with the expression plasmids for the rFVIII variants, editors, and gRNA, followed by the evaluation of secreted rFVIII levels (Figure 2B).

For the p.R2016W variant, except for the SaKKH-ABE8e/g1b combination, the ABE8e-NG/g1, Sp-PE2/peg1, and Sp-PE3/peg1 produced a significant increase of secreted rFVIII levels (from 4.9 ± 1.1 ng/mL to 14.3 ± 6.3 ng/mL, 13.1 ± 3.5 ng/mL, and 12.0 ± 3.0 ng/mL, respectively). Also, the xABE7.10/g1 resulted in increased, albeit not statistically significant, secreted levels (10.8 ± 4.7 ng/mL). For the p.R2166* nonsense variant, all editor systems tested resulted in a significant increase of secreted rFVIII levels in media, with the ABE8e-NG/g4 and ABE8e-NG/g4c displaying the highest correction effect (from <0.1 ng/mL to 13.7 ± 4.2 ng/mL and 9.5 ± 2.1 ng/mL, respectively). The p.R2182H change resulted to be efficiently rescued by the PE2-based system, with secreted rFVIII antigen levels rising from 11.9 ± 0.8 ng/mL to 16.7 ± 0.3 ng/mL. Lastly, the p.R2228Q variant was significantly rescued by all approaches tested (ABE8e-NG with g3 and Sp-PE2 with peg3 or peg3c from 2.0 ± 0.1 ng/mL to 4.3 ± 0.3 ng/mL, 12.2 ± 2.2 ng/mL, or 13.0 ± 1.3 ng/mL, respectively). Conversely, the ABE8e-NG with g3b was ineffective. For the p.R2182H and p.R2228Q variants, the Sp-PE3 was significantly more effective than Sp-PE2 in rescuing the targeted mutations (from 16.7 ± 0.3 ng/mL to 24.8 ± 4.4 ng/mL and from 12.2 ± 2.2 ng/mL to 18.9 ± 1.4 ng/mL, respectively).

To verify the impact of the rescue on the functional FVIII features, we carried out APTT-based assays in conditioned medium (Figure 2C).

For the p.R2016W variant, the increase in activity upon Sp-PE2/peg1 treatment was consistent with that in secreted protein levels (22.8 ± 4.1%), while the ABE8e-NG/g1 treatment did not impact FVIII activity (1.3 ± 0.2%).

For the p.R2166* variant, a remarkable functional rescue was obtained with the ABE8e-NG/g4 (39.7 ± 8.6%), while the impact of the Sp-PE2/peg4 was barely detectable (1.8 ± 1.4%). The p.R2182H was rescued by the Sp-PE3/peg2/g2 (29.4 ± 2.3%) but not with the ABE8e-NG/g2b. The p.R2228Q variant showed a clear functional rescue through either the ABE8e-NG/g3 or the Sp-PE3/peg3/g3 combinations (14.6 ± 0.1% and 48.4 ± 10.5%, respectively).

Overall, this screening phase led to the selection of a BE or PE approach for each model mutation that was able to efficiently rescue secretion of functional rFVIII.

3.2 | Correction efficiency of the selected BE and PE systems in stable rFVIII-expressing clones

To better appreciate the rescue, we generated stable clones expressing the rFVIII variants bearing the p.R2166* and p.R2228Q mutations chosen as paradigmatic examples of nonsense and missense mutations associated with the lowest secreted rFVIII levels and with the highest correction efficiency. Secreted rFVIII levels from stable clones were consistent with those observed in transient transfection experiments, with the mutations p.R2166* and p.R2228Q associated with no/barely detectable traces (<1%) and 7.7 ± 1.1% of rFVIII antigen level in cell media, respectively (Supplementary Figure S1). Analysis of rFVIII protein levels in cell lysates revealed reduced (~65% of WT) FVIII protein levels for both mutations. Moreover, proliferation assay revealed that clones expressing rFVIII mutants grew at significantly slower rate (~50%) compared with those expressing rFVIIIwt (Supplementary Figure S1), which could underlie endoplasmic reticulum stress triggered by unfolded protein [49].

In this experimental setting, the p.R2166* variant was approached with the ABE8e-NG and 2 different gRNAs and the ABEmax-NRTH with its own specific gRNA. As shown in Figure 3, the p.R2166* was significantly rescued by ABE8e-NG with both gRNAs at both antigen (from 1.4 ± 0.4% to 18.9 ± 1.3%; $P \leq .0001$ or 3.0 ± 0.7%; $P = .0093$, respectively) and activity levels (from <1% to 14.4 ± 5.0%; $P = .0112$ or 2.1 ± 0.3%; $P = .010$). Differently, ABEmax-NRTH variant-mediated rescue was poorly detectable.

The p.R2228Q variant was approached with the ABE8e-NG and with the PE2 options (Sp, SpRY, and SpG Cas9 variants), each with its own pegRNAs (Figure 3). In cotransfection experiments, ABE8e-NG, Sp-PE2, and SpG-PE2 rescued p.R2228Q at both antigen (from 7.7 ± 1.1% to 21.5 ± 7.0%; $P = .0279$; 34.8 ± 3.4%; $P = .0002$; and 17.2 ± 1.2%; $P \leq .0001$) and activity levels (from 6.3 ± 1.4% to 33.1 ± 10.9%; $P = .0499$; 36.7 ± 8.4%; $P = .0047$; and 16.1 ± 6.9%; $P = .0643$, respectively). Vice versa, the SpRY-PE2 variant was poorly effective, albeit associated with an increase in secreted FVIII levels.

To provide direct experimental evidence of the mutation reversion, we carried out DNA Sanger sequencing of the targeted gene regions, which revealed the desired A > G conversion for both FVIII variants treated with the BE/PE combinations associated with the most appreciable effect on secreted FVIII levels (Figure 4). Quantification of editing by relative peak height from 3 independent experiments led to estimate a correction efficiency of 29 ± 4% for the p.R2166* change with the ABE8e-NG/g4 and of 27 ± 3% or 21 ± 4%

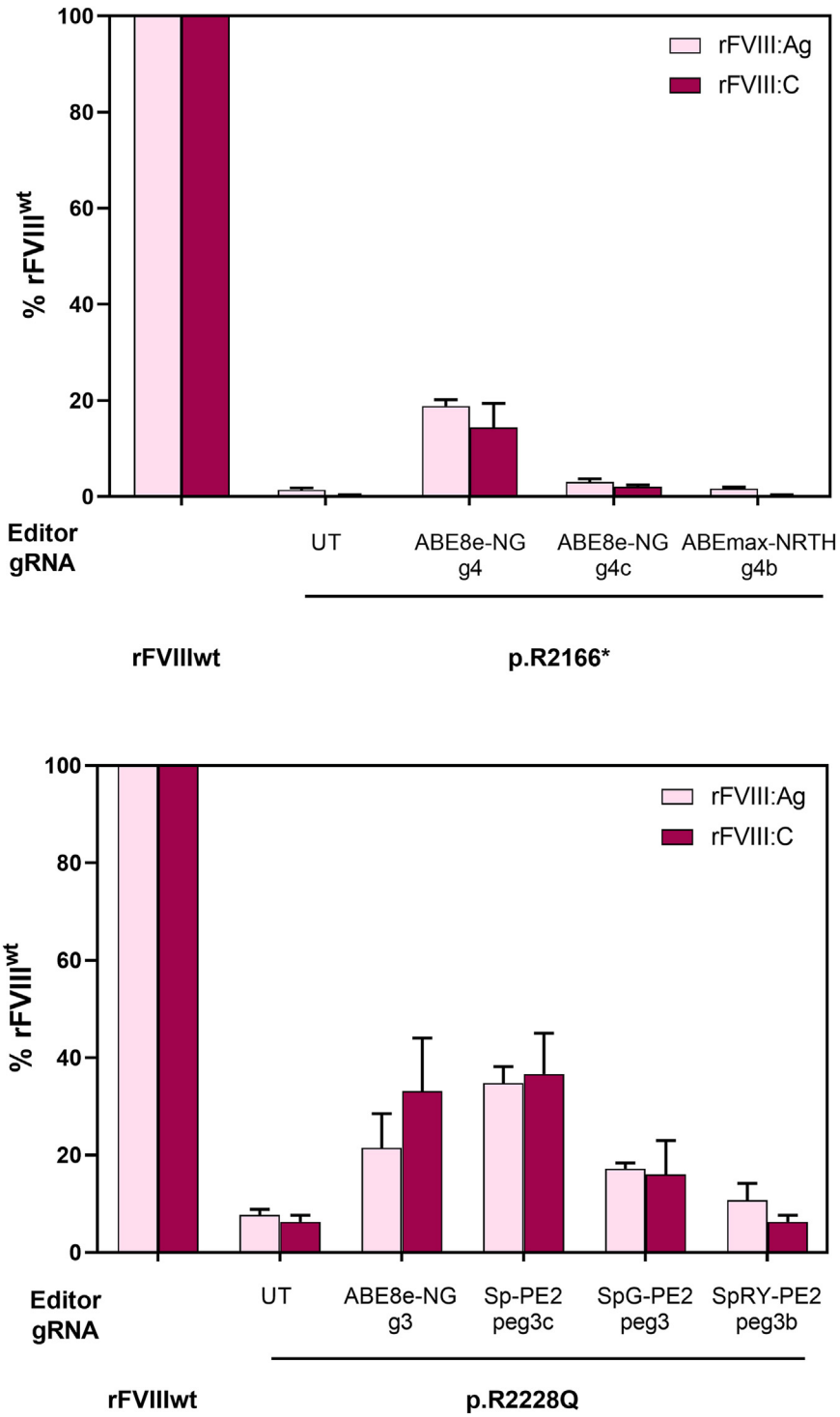


FIGURE 3 Base editor (BE)/prime editor (PE)-mediated rescue of recombinant factor VIII (rFVIII) antigen (rFVIII:Ag) and activity (rFVIII:C) levels in stable clones. rFVIII levels in stable human embryonic kidney 293T clones expressing the wild-type (rFVIIIwt) or mutant rFVIII cassettes alone (UT) or upon treatment with the selected BEs/PEs. The SpRY and SpG PE tools, made available during the study, were directly tested in the stable clone. rFVIII:Ag and rFVIII:C levels are reported as the percentage of rFVIIIwt. Results are presented as mean ± SD of 3 independent experiments. *$P < .1$; **$P < .01$; ***$P < .001$; ****$P < .0001$. ABE, adenine base editor; gRNA, guide RNA; pegRNA, prime editing guide RNA; UT, untreated.

for the p.R2228Q mutation with the ABE8e-NG/g3 or Sp-PE2/peg3c, respectively. Sequencing of the p.R2166* mutant targeted with the ABE8e-NG/g4 also revealed a bystander G to A editing (p.Y2167H) with an efficiency of 38 ± 11%. Altogether, these data demonstrate that the selected best-performing BE and PE combination can efficiently revert the target mutations, thus ultimately rescuing the secretion of functional FVIII.

3.3 | Rescue of FVIII in *ex vivo* BOECs by lentiviral delivery of ABE8e-NG

Data from stable clones prompted us to attempt the correction of both FVIII variants in the model of human BOECs, lately called endothelial progenitor cells, isolated from both peripheral blood and considered a viable *ex vivo* cellular model for cell and gene therapy for

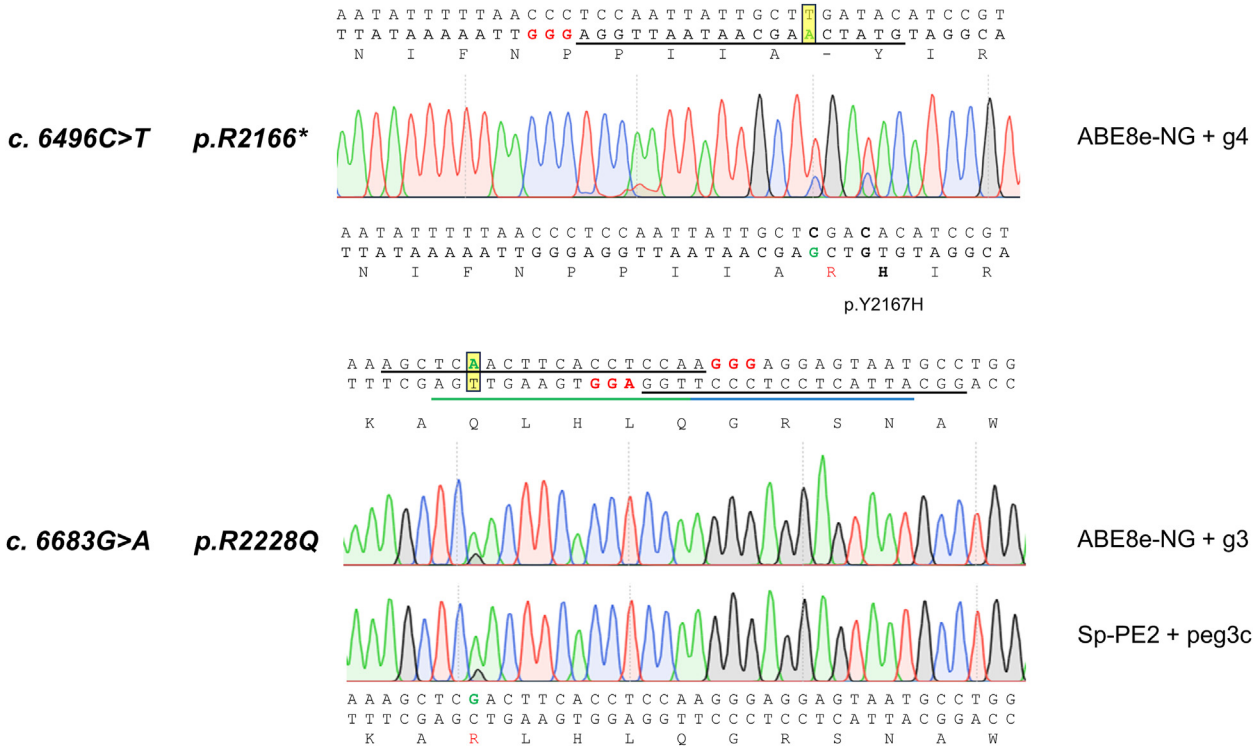


FIGURE 4 Base editor/prime editor-mediated correction of causative Hemophilia A mutations at DNA level. Representative electropherograms from DNA sequencing of the targeted region in the stable clones that, upon treatment with base editors/prime editors, showed the highest rescue of recombinant factor VIII secretion and function. The red and underlined letters indicate the protospacer adjacent motif and guide RNA sequences, respectively. The primer binding site and reverse transcriptase template sequences of prime editing guide RNA are indicated by blue and green underlined letters, respectively. The targeted mutation, before and after editing, is reported in green and in the upper and lower parts of the electropherograms, respectively. The amino acid change resulting from the editing is reported in red. ABE, adenine base editor.

HA [48,50–52]. Since BOECs isolated from HA patients with the mutations under investigation were not available, we generated new models of BOECs consisting of BOECs from an HA patient harboring the *FVIII* IVS22 mutation and not producing appreciable secreted FVIII levels, transduced with an LV expressing the FVIII variant under investigation (Figure 5A). As shown in Figure 5B, C, BOECs transduced with the LV-FVIII-R2166* or LV-FVIII-R2228Q mimicked what was previously detected in stable FVIII clones, with p.R2166* and p.R2228Q associated with undetectable and low levels of functional FVIII levels, respectively. On the other hand, the secreted FVIII from BOECs transduced with LV-FVIIIwt, created as an additional control, shortened the FIX-dependent APTT-based coagulation time from 167.8 ± 6.7 seconds to 46.9 ± 4.2 seconds, corresponding to 30 ng/mL.

To trigger editing, we selected the ABE8e-NG since the coding cassette of ABE is smaller than the PE counterpart and, therefore more suitable to be efficiently loaded and delivered via LV.

BOECs were then transduced at different MOIs (from 1 to 20) by LVs expressing the ABE8e-NG and the 2 mutation-specific gRNAs. The evaluation of FVIII antigen and activity levels in cell media 3-day posttreatment revealed a significant and dose-dependent rescue of rFVIII for both mutations. In particular, rFVIII antigen levels increased from 0 to 17.4 ± 0.6 ng/mL (p.R2166*) and 32.9 ± 2 ng/mL (p.R2228Q) at MOI 20 (Figure 5B), which were paralleled by a concurrent decrease in coagulation time (from 167.8 ± 6.7 seconds to 52.8 ± 0.8 seconds for p.R2166*; from 151.9 ± 14.7 seconds to 42.5 ± 2.5 seconds for p.R2228Q). Analysis of rFVIII-specific activity showed that for both mutations, rescued FVIII possesses a roughly normal specific activity close to that observed for the rFVIIIwt (Figure 5C).

Overall, these data demonstrated that LV-mediated delivery of ABE8e-NG with the mutation-specific gRNA can efficiently rescue secretion of a functional FVIII protein in *ex vivo* BOECs.

4 | DISCUSSION

While the intense research over decades has provided HA patients with a plethora of therapeutic and prophylactic options that remarkably reduced bleeding tendency and ameliorated the quality of life [6], a definitive cure is not available yet. To this purpose, enormous progresses have been made with additive gene therapy based on adeno-associated viral vectors, with valoctocogene roxaparvovec that recently received authorization from regulatory agencies, but several open issues still have to be addressed, including the current restriction on adults [9,14,15,53]. Conversely, the correction of the defect detected in the patient's *FVIII* gene would provide a definitive cure, particularly in replicative tissues such as liver of young patients.

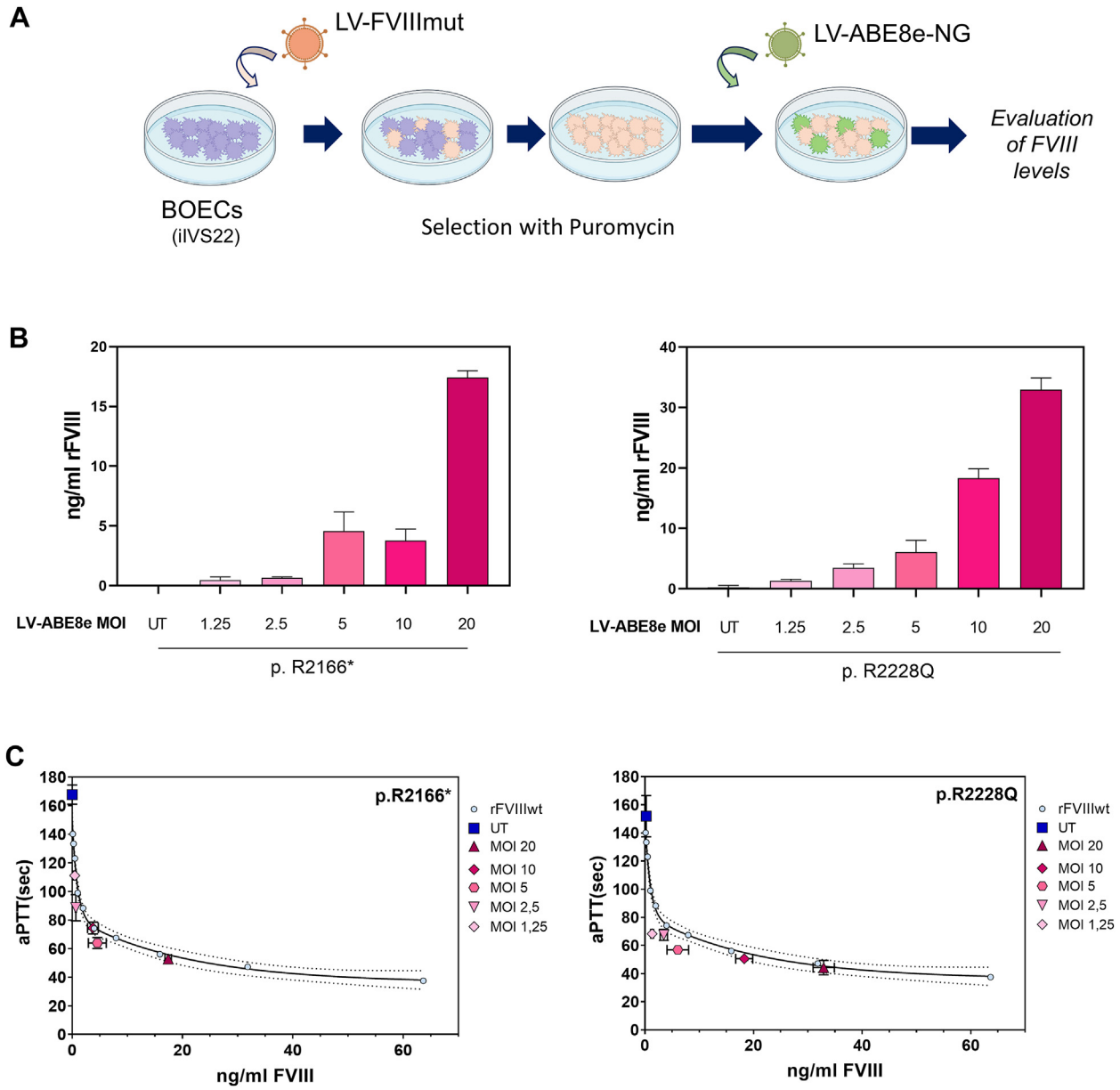


FIGURE 5 Base Editor (BE)-mediated correction of causative hemophilia A mutations in blood outgrowth endothelial cell (BOEC) cellular models. (A) Schematic representation of the protocol exploited for the generation of mutation-specific BOEC models and evaluation of BE-mediated correction through lentiviral vector (LV)-mediated delivery of ABE8e editors. See Results for experimental details. (B) Recombinant factor VIII (rFVIII) antigen levels in media from BOECs transduced with LV expressing the mutation-specific ABE8e editor at different multiplicity of infection (MOI). Results are presented as mean ± SD of 2 independent experiments. Higher MOIs are reported with increasing saturated violet color. (C) rFVIII coagulant activity in media from BOECs transduced with LVs expressing the mutation-specific ABE8e editor at different MOI. rFVIII antigen and coagulation time are reported on the x- and y-axes, respectively. A standard curve made with wild-type rFVIII (rFVIIIwt, light blue circles) from stable clone is reported. Higher MOIs are reported with increasing saturated violet color. Results are presented as mean ± SD of 2 independent experiments. ABE, adenine base editor; APTT, activated partial thromboplastin time; UT, untreated.

So far, extensive research has been conducted to trigger HR through nuclease-induced DSB [20], which still suffers from low efficiency and poses safety concerns related to potential off-target DSBs.

For these reasons, the recently developed DNA editing systems such as BE and PE systems, not relying on DSB and HR, represent attractive approaches to efficiently and permanently revert point mutations, which are the most frequent cause of human disease [54]. Because of the high efficiency of BE, the extreme versatility of PE, and the limited off-target effects, BE and PE have been so far successfully applied in a variety of cell types and organisms, including animal models of human genetic diseases [55,56]. BE is now close to the clinic for heterozygous familial hypercholesterolemia and severe sickle cell disease [57,58]. Despite these promising results, very few attempts have been made with BE or PE in hemorrhagic coagulation factor

disorders where even a partial rescue of functional plasma levels would remarkably ameliorate the clinical phenotype. To date, only the BE has been attempted in hemophilia B and proved to revert 2 *F9* missense mutations [59,60].

In this pioneering project, we explored for the first time the BE and PE approaches on *FVIII* point mutations, accounting for over 30% of all severe HA patients, the large majority of which are theoretically correctable by exploiting the variety of BE and PE systems so far developed (Supplementary Table S3).

Through *in vitro* and *ex vivo* studies on paradigmatic examples of missense and nonsense model mutations associated with severe bleeding phenotypes, we provided proof of principle of the therapeutic potential of BE/PE approaches for HA. FVIII antigen and activity of the recombinant variants in medium were mostly consistent with the coagulation phenotype in patients, with undetectable levels for p.R2166* nonsense mutation, major (p.R2228Q) or moderate (p.R2182H) impairment of secretion, and a combined detrimental effect on both secretion and activity for the p.R2016W, the latter confirming our previous characterization [61]. The *in vitro* system, which does not permit the mutation impact on FVIII stability and/or removal from the circulation, could in part explain the not uncommon discrepancy between FVIII levels reported in patients and recombinant data for the p.R2182H variant.

For each mutation, the initial screening phase in transient transfection experimental systems led to the identification of a short panel of BE/gRNA and PE/pegRNA combinations able to efficiently rescue FVIII expression. In particular, the ABE8e-NG and Sp-PE3 were confirmed to be the most active in the editing compared with the ABE7.10, xABE, or Sp-PE2 [33,62]. It is worth noting that some combinations (ie, Sp-PE2/PE3 for the p.R2166* or xABE7.10/g2 for the p.R2182H variant) were ineffective or led to a further reduction of the secreted FVIII levels, which might be attributable to bystander effects of BEs. The same mechanism might lead to rescue secretion but not activity as in the case of the p.R2016W variant targeted by the ABE8e/g1 combination. These data highlight the importance of accurate computational design of the specific correction approaches (BE or PE), followed by extensive experimental screening, to select those promoting effective editing.

The selected BE- and PE-based combinations were successfully exploited in cells stably expressing the selected nonsense p.R2166* and the missense p.R2228Q because they were found to be associated with very low secreted FVIII and displaying the highest correction efficiency. Analysis of the rescued FVIII expression corroborated findings in the transient transfection assays with an overall correction efficiency of 20% to 30% for both mutations. Noticeably, the consistent increase of the FVIII antigen and activity levels pointed toward a normal specific activity of the rescued FVIII, suggesting an efficient conversion of the point mutation to the WT counterpart. This was clearly demonstrated by DNA sequencing of the targeted gene region selected by allele-specific PCR. More specifically, the analysis of sequence trace decomposition showed an editing efficiency of 21% to 29%, an extent consistent with the rescued FVIII antigen and activity levels. On the other hand, sequencing revealed an undesired adjacent editing for the p.R2166* mutation targeted by the ABE8e-NG/g4 combination, which occurred with comparable efficiency (~38%) to that of the correct one (~30%). This would lead to the introduction of the p.Y2167H change, whose effect is predicted to be detrimental. However, the rescue of the secreted and functional FVIII levels (~20% vs ~30% of mutation reversion) points toward the occurrence, in the majority of cases, of 2 distinct editing events: i) the candidate mutation reversion only and the rescue impact, or ii) the bystander effect, with the introduction of the p.Y2167H substitution in the null p.R2166* allele, which does not cause additional harm. Although the safer profile of BE/PE is compared with that of nuclease-mediated editing approaches [63], we recognize the need for extensive genome-wide evaluation to assess off-target effects of our FVIII-tailored combinations, which are hard to bioinformatically predict at present.

The successful editing was further demonstrated in BOECs, which better recapitulate the physiological expression site for FVIII. To overcome the unavailability of BOEC models harboring the mutations under investigation and to avoid the potential confounding effect of endogenous FVIII produced by WT BOECs [48], we engineered BOECs from an HA patient with the *FVIII* IVS22 inversion and not expressing appreciable FVIII levels. By LV, we integrated the FVIII coding cassette for the 2 model mutations into BOEC^{IVS22inv} and demonstrated that both cellular models recapitulated findings from our previous cellular models, with the p.R2166* and p.R2228Q variants associated with undetectable and barely detectable levels of rFVIII, respectively. By recognizing that these *ex vivo* HA cellular models may not fully recapitulate conditions (ie, transgene integration position and chromatin context) that might be relevant for the editing process *ex vivo* or *in vivo*, the treatment with the ABE8e-based BE resulted in a remarkable and dose-dependent increase of secreted FVIII. This was paralleled by the shortening of the coagulation time in APTT-based FVIII activity assays that, at the highest dose, mirrored the functional levels expressed by BOECs transduced with the LV-FVIIIwt.

In conclusion, these data provide the experimental proof-of-concept that delivery of tailored BEs and PEs can effectively revert pathogenic and common HA single nucleotide mutations at the DNA level, thus leading to appreciable rescue of production and secretion of functional FVIII. It is tempting to speculate that, if translated in patients, the extent of rescue obtained with a single intervention would remarkably ameliorate the coagulation and clinical phenotype with lifelong effects, even in young patients. When considering the editing windows and the protospacer adjacent motif constraints, the BE/PE approaches could target up to 90% of point mutations, with therapeutic implications for up to 97% of patients bearing these nucleotide changes (Supplementary Table S3).

It is worth noting that these therapeutic approaches could take advantage of nonviral gene delivery systems [59,64,65], preventing the persistence of the editors and thus further minimizing off-target effects. This might be envisaged for *ex vivo* gene therapy purposes

(ie, endothelial cell progenitor cells) [48] or direct *in vivo* FVIII gene editing in liver sinusoidal endothelial cells taking advantage of endothelial-specific promoters (ie, stabilin-2) [66].

Altogether, these results lay the foundation for safety and efficacy studies in animal models with specific mutations, not yet available, with BEs and PEs to develop a versatile and personalized definitive cure for large cohorts of HA patients.

ACKNOWLEDGMENTS

This work was supported by grants to M.P. from Bayer (Bayer Hemophilia Award Program BHAP 2020) and to D.B. from Novo Nordisk (Novo Nordisk Access to Insight Grants Program 2022) and Associazione Italiana Centri Emofilia (AICE). A.F. was supported by Telethon grant number GGP19201. The schematic illustrations were created with BioRender.com. We would like to thank Kevin Zhao, Tony Huang, and David Liu, all from Harvard University, Cambridge, Massachusetts, United States, for providing valuable suggestions.

AUTHOR CONTRIBUTIONS

E.T. performed bioinformatic analyses and research and interpreted data; A.C. performed experiments and interpreted data in blood outgrowth endothelial cells; M.P. and D.B. conceived the study, supervised research, and coordinated the work; M.P., D.B., A.F., and F.B. wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

ORCID

Dario Balestra https://orcid.org/0000-0002-6675-9429

REFERENCES

[1] White GC 2nd, Rosendaal F, Aledort LM, Lusher JM, Rothschild C, Ingerslev J, Factor VIII, Subcommittee Factor IX. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost*. 2001;85:560.

[2] Do H, Healey JF, Waller EK, Lollar P. Expression of factor VIII by murine liver sinusoidal endothelial cells. *J Biol Chem*. 1999;274:19587–92.

[3] Follenzi A, Benten D, Novikoff P, Faulkner L, Raut S, Gupta S. Transplanted endothelial cells repopulate the liver endothelium and correct the phenotype of hemophilia A mice. *J Clin Invest*. 2008;118:935–45.

[4] Zanolini D, Merlin S, Feola M, Ranaldo G, Amoruso A, Gaidano G, Zaffaroni M, Ferrero A, Brunelleschi S, Valente G, Gupta S, Prat M, Follenzi A. Extrahepatic sources of factor VIII potentially contribute to the coagulation cascade correcting the bleeding phenotype of mice with hemophilia A. *Haematologica*. 2015;100:881–92.

[5] Konkle BA. Impacting inhibitor development in hemophilia A. *Blood*. 2017;130:1689–90.

[6] Mannucci PM. Hemophilia treatment innovation: 50 years of progress and more to come. *J Thromb Haemost*. 2023;21:403–12.

[7] Knoebl P, Thaler J, Jilma P, Quehenberger P, Gleixner K, Sperr WR. Emicizumab for the treatment of acquired hemophilia A. *Blood*. 2021;137:410–9.

[8] Butterfield JSS, Hege KM, Herzog RW, Kaczmarek R. A molecular revolution in the treatment of hemophilia. *Mol Ther*. 2020;28:997–1015.

[9] Ozelo MC, Mahlangu J, Pasi KJ, Giermasz A, Leavitt AD, Laffan M, Symington E, Quon DV, Wang JD, Peerlinck K, Pipe SW, Madan B, Key NS, Pierce GF, O'Mahony B, Kaczmarek R, Henshaw J, Lawal A, Jayaram K, Huang M, et al. Valoctocogene roxaparvovec gene therapy for hemophilia A. *N Engl J Med*. 2022;386:1013–25.

[10] Mahlangu J, Kuliczkowski K, Karim FA, Stasyshyn O, Kosinova MV, Lepatan LM, Skotnicki A, Boggio LN, Klamroth R, Oldenburg J, Hellmann A, Santagostino E, Baker RI, Fischer K, Gill JC, P'Ng S, Chowdary P, Escobar MA, Khayat CD, Rusen L, et al. Efficacy and safety of rVIII-SingleChain: results of a phase 1/3 multicenter clinical trial in severe hemophilia A. *Blood*. 2016;128:630–7.

[11] George LA, Monahan PE, Eyster ME, Sullivan SK, Ragni MV, Croteau SE, Rasko JEJ, Recht M, Samelson-Jones BJ, MacDougall A, Jaworski K, Noble R, Curran M, Kuranda K, Mingozzi F, Chang T, Reape KZ, Anguela XM, High KA. Multiyear factor VIII expression after AAV gene transfer for hemophilia A. *N Engl J Med*. 2021;385:1961–73.

[12] Leavitt AD, Konkle BA, Stine K, Visweshwar N, Harrington TJ, Giermasz A, Arkin S, Fang A, Plonski F, Smith L, Tseng LJ, Di Russo G, Cockroft BM, Rupon J, Rouy D. Updated follow-up of the Alta Study, a phase 1/2 study of giroctocogene fitelparvovec (SB-525) gene therapy in adults with severe hemophilia A. *Blood*. 2020;136:12.

[13] Pasi KJ, Rangarajan S, Mitchell N, Lester W, Symington E, Madan B, Laffan M, Russell CB, Li M, Pierce GF, Wong WY. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. *N Engl J Med*. 2020;382:29–40.

[14] Colella P, Ronzitti G, Mingozzi F. Emerging issues in AAV-mediated in vivo gene therapy. *Mol Ther Methods Clin Dev*. 2018;8:87–104.

[15] Mendell JR, Al-Zaidy SA, Rodino-Klapac LR, Goodspeed K, Gray SJ, Kay CN, Boye SL, Boye SE, George LA, Salabarria S, Corti M, Byrne BJ, Tremblay JP. Current clinical applications of in vivo gene therapy with AAVs. *Mol Ther*. 2021;29:464–88.

[16] Wu Y, Hu Z, Li Z, Pang J, Feng M, Hu X, Wang X, Lin-Peng S, Liu B, Chen F, Wu L, Liang D. In situ genetic correction of F8 intron 22 inversion in hemophilia A patient-specific iPSCs. *Sci Rep*. 2016;6:18865.

[17] Lansing F, Mukhametzyanova L, Rojo-Romanos T, Iwasawa K, Kimura M, Paszkowski-Rogacz M, Karpinski J, Grass T, Sonntag J, Schneider PM, Günes C, Hoersten J, Schmitt LT, Rodriguez-Muela N, Knöfler R, Takebe T, Buchholz F. Correction of a factor VIII genomic inversion with designer-recombinases. *Nat Commun*. 2022;13:422.

[18] Sung JJ, Park CY, Leem JW, Cho MS, Kim DW. Restoration of FVIII expression by targeted gene insertion in the FVIII locus in hemophilia A patient-derived iPSCs. *Exp Mol Med*. 2019;51:1–9.

[19] Sharma R, Anguela XM, Doyon Y, Wechsler T, DeKelver RC, Sproul S, Paschon DE, Miller JC, Davidson RJ, Shivak D, Zhou S, Rieders J, Gregory PD, Holmes MC, Rebar EJ, High KA. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. *Blood*. 2015;126:1777–84.

[20] Hu Z, Wu Y, Xiao R, Zhao J, Chen Y, Wu L, Zhou M, Liang D. Correction of F8 intron 1 inversion in hemophilia A patient-specific iPSCs by CRISPR/Cas9 mediated gene editing. *Front Genet*. 2023;14:1115831.

[21] Abudayyeh OO, Gootenberg JS, Franklin B, Koob J, Kellner MJ, Ladha A, Joung J, Kirchgatterer P, Cox DBT, Zhang F. A cytosine deaminase for programmable single-base RNA editing. *Science*. 2019;365:382–6.

[22] Rees HA, Liu DR. Base editing: precision chemistry on the genome and transcriptome of living cells. *Nat Rev Genet*. 2018;19:770–88.

[23] Gehrke JM, Cervantes O, Clement MK, Wu Y, Zeng J, Bauer DE, Pinello L, Joung JK. An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities. *Nat Biotechnol*. 2018;36:977–82.

[24] Koblan LW, Doman JL, Wilson C, Levy JM, Tay T, Newby GA, Maianti JP, Raguram A, Liu DR. Improving cytidine and adenine base editors by expression optimization and ancestral reconstruction. *Nat Biotechnol*. 2018;36:843–6.
[25] Tan J, Zhang F, Karcher D, Bock R. Engineering of high-precision base editors for site-specific single nucleotide replacement. *Nat Commun*. 2019;10:439.
[26] Levy JM, Yeh WH, Pendse N, Davis JR, Hennessey E, Butcher R, Koblan LW, Comander J, Liu Q, Liu DR. Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses. *Nat Biomed Eng*. 2020;4:97–110.
[27] Kim Y, Hong SA, Yu J, Eom J, Jang K, Yoon S, Hong DH, Seo D, Lee SN, Woo JS, Jeong J, Bae S, Choi D. Adenine base editing and prime editing of chemically derived hepatic progenitors rescue genetic liver disease. *Cell Stem Cell*. 2021;28:1614–24.e5.
[28] Suh S, Choi EH, Leinonen H, Foik AT, Newby GA, Yeh WH, Dong Z, Kiser PD, Lyon DC, Liu DR, Palczewski K. Restoration of visual function in adult mice with an inherited retinal disease via adenine base editing. *Nat Biomed Eng*. 2021;5:169–78.
[29] Jang HK, Jo DH, Lee SN, Cho CS, Jeong YK, Jung Y, Yu J, Kim JH, Woo JS, Bae S. High-purity production and precise editing of DNA base editing ribonucleoproteins. *Sci Adv*. 2021;7:eabg2661.
[30] Ryu SM, Koo T, Kim K, Lim K, Baek G, Kim ST, Kim HS, Kim D, Lee H, Chung E, Kim JS. Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy. *Nat Biotechnol*. 2018;36:536–9.
[31] Rothgangl T, Dennis MK, Lin PJC, Oka R, Witzigmann D, Villiger L, Qi W, Hruzova M, Kissling L, Lenggenhager D, Borrelli C, Egli S, Frey N, Bakker N, Walker JA 2nd, Kadina AP, Victorov DV, Pacesa M, Kreutzer S, Kontarakis Z, et al. In vivo adenine base editing of PCSK9 in macaques reduces LDL cholesterol levels. *Nat Biotechnol*. 2021;39:949–57.
[32] Gillmore JD, Gane E, Taubel J, Kao J, Fontana M, Maitland ML, Seitzer J, O'Connell D, Walsh KR, Wood K, Phillips J, Xu Y, Amaral A, Boyd AP, Cehelsky JE, McKee MD, Schiermeier A, Harari O, Murphy A, Kyratsous CA, et al. CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. *N Engl J Med*. 2021;385:493–502.
[33] Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, Chen PJ, Wilson C, Newby GA, Raguram A, Liu DR. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*. 2019;576:149–57.
[34] Chen PJ, Hussmann JA, Yan J, Knipping F, Ravisankar P, Chen PF, Chen C, Nelson JW, Newby GA, Sahin M, Osborn MJ, Weissman JS, Adamson B, Liu DR. Enhanced prime editing systems by manipulating cellular determinants of editing outcomes. *Cell*. 2021;184:5635–52.e29.
[35] Doman JL, Pandey S, Neugebauer ME, An M, Davis JR, Randolph PB, McElroy A, Gao XD, Raguram A, Richter MF, Everette KA, Banskota S, Tian K, Tao YA, Tolar J, Osborn MJ, Liu DR. Phage-assisted evolution and protein engineering yield compact, efficient prime editors. *Cell*. 2023;186:3983–4002.e26.
[36] Habib O, Habib G, Hwang GH, Bae S. Comprehensive analysis of prime editing outcomes in human embryonic stem cells. *Nucleic Acids Res*. 2022;50:1187–97.
[37] McVey JH, Rallapalli PM, Kemball-Cook G, Hampshire DJ, Giansily-Blaizot M, Gomez K, Perkins SJ, Ludlam CA. The European Association for Haemophilia and Allied Disorders (EAHAD) Coagulation Factor Variant Databases: important resources for haemostasis clinicians and researchers. *Haemophilia*. 2020;26:306–13.
[38] Lakich D, Kazazian HH, Antonarakis SE, Gitschier J. Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. *Nat Genet*. 1993;5:236–41.
[39] Bagnall RD, Waseem N, Green PM, Giannelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood*. 2002;99:168–74.
[40] Hwang GH, Jeong YK, Habib O, Hong SA, Lim K, Kim JS, Bae S. PE-designer and PE-analyzer: web-based design and analysis tools for CRISPR prime editing. *Nucleic Acids Res*. 2021;49:W499–504.
[41] Hwang GH, Bae S. Web-based computational tools for base editors. *Methods Mol Biol*. 2023;2606:13–22.
[42] Anderson MV, Haldrup J, Thomsen EA, Wolff JH, Mikkelsen JG. pegIT - a web-based design tool for prime editing. *Nucleic Acids Res*. 2021;49:W505–9.
[43] Chow RD, Chen JS, Shen J, Chen S. A web tool for the design of prime-editing guide RNAs. *Nat Biomed Eng*. 2021;5:190–4.
[44] Hwang GH, Park J, Lim K, Kim S, Yu J, Yu E, Kim ST, Eils R, Kim JS, Bae S. Web-based design and analysis tools for CRISPR base editing. *BMC Bioinformatics*. 2018;19:542.
[45] Arbab M, Shen MW, Mok B, Wilson C, Matuszek Z, Cassa CA, Liu DR. Determinants of base editing outcomes from target library analysis and machine learning. *Cell*. 2020;182:463–80.e30.
[46] Concordet JP, Haeussler M. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res*. 2018;46:W242–5.
[47] Kluesner MG, Nedveck DA, Lahr WS, Garbe JR, Abrahante JE, Webber BR, Moriarity BS. EditR: a method to quantify base editing from Sanger sequencing. *CRISPR J*. 2018;1:239–50.
[48] Olgasi C, Borsotti C, Merlin S, Bergmann T, Bittorf P, Adewoye AB, Wragg N, Patterson K, Calabria A, Benedicenti F, Cucci A, Borchiellini A, Pollio B, Montini E, Mazzuca DM, Zierau M, Stolzing A, Toleikis PM, Braspenning J, Follenzi A. Efficient and safe correction of hemophilia A by lentiviral vector-transduced BOECs in an implantable device. *Mol Ther Methods Clin Dev*. 2021;23:551–66.
[49] Poothong J, Pottekat A, Siirin M, Campos AR, Paton AW, Paton JC, Lagunas-Acosta J, Chen Z, Swift M, Volkmann N, Hanein D, Yong J, Kaufman RJ. Factor VIII exhibits chaperone-dependent and glucose-regulated reversible amyloid formation in the endoplasmic reticulum. *Blood*. 2020;135:1899–911.
[50] Lin Y, Chang L, Solovey A, Healey JF, Lollar P, Hebbel RP. Use of blood outgrowth endothelial cells for gene therapy for hemophilia A. *Blood*. 2002;99:457–62.
[51] Ozelo MC, Vidal B, Brown C, Notley C, Hegadorn C, Webster S, Harpell L, Ahlin J, Winterborn A, Handforth J, Arruda VR, Hough C, Lillicrap D. Omental implantation of BOECs in hemophilia dogs results in circulating FVIII antigen and a complex immune response. *Blood*. 2014;123:4045–53.
[52] Lyons CJ, O'Brien T. The functionality of endothelial-colony-forming cells from patients with diabetes mellitus. *Cells*. 2020;9:1731.
[53] Mahlangu J, Kaczmarek R, Von Drygalski A, Shapiro S, Chou SC, Ozelo MC, Kenet G, Peyvandi F, Wang M, Madan B, Key NS, Laffan M, Dunn AL, Mason J, Quon DV, Symington E, Leavitt AD, Oldenburg J, Chambost H, Reding MT, et al. Two-year outcomes of valoctocogene roxaparvovec therapy for hemophilia A. *N Engl J Med*. 2023;388:694–705.
[54] Stenson PD, Ball EV, Mort M, Phillips AD, Shiel JA, Thomas NST, Abeysinghe S, Krawczak M, Cooper DN. Human Gene Mutation Database (HGMD): 2003 update. *Hum Mutat*. 2003;21:577–81.
[55] Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime. *Nat Biotechnol*. 2020;38:824–44.
[56] Porto EM, Komor AC, Slaymaker IM, Yeo GW. Base editing: advances and therapeutic opportunities. *Nat Rev Drug Discov*. 2020;19:839–59.

[57] Musunuru K, Chadwick AC, Mizoguchi T, Garcia SP, DeNizio JE, Reiss CW, Wang K, Iyer S, Dutta C, Clendaniel V, Amaonye M, Beach A, Berth K, Biswas S, Braun MC, Chen HM, Colace TV, Ganey JD, Gangopadhyay SA, Garrity R, et al. In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates. *Nature*. 2021;593:429–34.

[58] Gaudelli NM, Lam DK, Rees HA, Solá-Esteves NM, Barrera LA, Born DA, Edwards A, Gehrke JM, Lee SJ, Liquori AJ, Murray R, Packer MS, Rinaldi C, Slaymaker IM, Yen J, Young LE, Ciaramella G. Directed evolution of adenine base editors with increased activity and therapeutic application. *Nat Biotechnol*. 2020;38:892–900.

[59] Rong L, Chen D, Huang X, Sun L. Delivery of Cas9-guided ABE8e into stem cells using poly(l-lysine) polypeptides for correction of the hemophilia-associated FIX missense mutation. *Biochem Biophys Res Commun*. 2022;628:49–56.

[60] Hiramoto T, Kashiwakura Y, Hayakawa M, Baatartsogt N, Kamoshita N, Abe T, Inaba H, Nishimasu H, Uosaki H, Hanazono Y, Nureki O, Ohmori T. PAM-flexible Cas9-mediated base editing of a hemophilia B mutation in induced pluripotent stem cells. *Commun Med (Lond)*. 2023;3:56.

[61] Donadon I, McVey JH, Garagiola I, Branchini A, Mortarino M, Peyvandi F, Bernardi F, Pinotti M. Clustered F8 missense mutations cause hemophilia A by combined alteration of splicing and protein biosynthesis and activity. *Haematologica*. 2018;103:344–50.

[62] Neugebauer ME, Hsu A, Arbab M, Krasnow NA, McElroy AN, Pandey S, Doman JL, Huang TP, Raguram A, Banskota S, Newby GA, Tolar J, Osborn MJ, Liu DR. Evolution of an adenine base editor into a small, efficient cytosine base editor with low off-target activity. *Nat Biotechnol*. 2023;41:673–85.

[63] Fiumara M, Ferrari S, Omer-Javed A, Beretta S, Albano L, Canarutto D, Varesi A, Gaddoni C, Brombin C, Cugnata F, Zonari E, Naldini MM, Barcella M, Gentner B, Merelli I, Naldini L. Genotoxic effects of base and prime editing in human hematopoietic stem cells. *Nat Biotechnol*. 2023. https://doi.org/10.1038/s41587-023-01915-4. in press.

[64] O'Keeffe Ahern J, Lara-Sáez I, Zhou D, Murillas R, Bonafont J, Mencía Á, García M, Manzanares D, Lynch J, Foley R, Xu Q, Sigen A, Larcher F, Wang W. Non-viral delivery of CRISPR-Cas9 complexes for targeted gene editing via a polymer delivery system. *Gene Ther*. 2022;29:157–70.

[65] Yin H, Song CQ, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, Gupta A, Bolukbasi MF, Walsh S, Bogorad RL, Gao G, Weng Z, Dong Y, Koteliansky V, Wolfe SA, Langer R, et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat Biotechnol*. 2016;34:328–33.

[66] Borroni E, Borsotti C, Cirsmaru RA, Kalandadze V, Famà R, Merlin S, Brown B, Follenzi A. Immune tolerance promotion by LSEC-specific lentiviral vector-mediated expression of the transgene regulated by the stabilin-2 promoter. *Mol Ther Nucleic Acids*. 2024;35:102116.

SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2024.04.020