

Defining the Optimal FVIII Transgene for Placental Cell-Based Gene Therapy to Treat Hemophilia A

Nadia El-Akabawy,^{1,2,6} Martin Rodriguez,^{1,6} Ritu Ramamurthy,¹ Andrew Rabah,¹ Brady Trevisan,¹ Alshaimaa Morsi,^{1,2} Sunil George,¹ Jordan Shields,^{3,4} Diane Meares,⁵ Andrew Farland,⁵ Anthony Atala,¹ Christopher B. Doering,^{3,4} H. Trent Spencer,^{3,4} Christopher D. Porada,^{1,7} and Graça Almeida-Porada^{1,7}

¹Wake Forest Institute for Regenerative Medicine, Fetal Research and Therapy Program, Wake Forest School of Medicine, Winston-Salem, NC 27101, USA; ²Zagazig University Faculty of Medicine, Zagazig, Egypt; ³Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, GA, USA; ⁴Department of Pediatrics, Emory University, Atlanta, GA, USA; ⁵Special Hematology Laboratory, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

The delivery of factor VIII (FVIII) through gene and/or cellular platforms has emerged as a promising hemophilia A treatment. Herein, we investigated the suitability of human placental cells (PLCs) as delivery vehicles for FVIII and determined an optimal FVIII transgene to produce/secrete therapeutic FVIII levels from these cells. Using three PLC cell banks we demonstrated that PLCs constitutively secreted low levels of FVIII, suggesting their suitability as a transgenic FVIII production platform. Furthermore, PLCs significantly increased FVIII secretion after transduction with a lentiviral vector (LV) encoding a myeloid codon-optimized bioengineered FVIII containing high-expression elements from porcine FVIII. Importantly, transduced PLCs did not upregulate cellular stress or innate immunity molecules, demonstrating that after transduction and FVIII production/secretion, PLCs retained low immunogenicity and cell stress. When LV encoding five different bioengineered FVIII transgenes were compared for transduction efficiency, FVIII production, and secretion, data showed that PLCs transduced with LV encoding hybrid human/porcine FVIII transgenes secreted substantially higher levels of FVIII than did LV encoding B domain-deleted human FVIII. In addition, data showed that in PLCs, myeloid codon optimization is needed to increase FVIII secretion to therapeutic levels. These studies have identified an optimal combination of FVIII transgene and cell source to achieve clinically meaningful levels of secreted FVIII.

INTRODUCTION

Hemophilia A (HA) therapies have advanced rapidly during the last decade;¹ among these, factor VIII (FVIII) transgene-modified cellular platforms have emerged as a new class of promising biologicals.²⁻⁴ The direct infusion of FVIII-encoding adeno-associated virus (AAV) vectors has yielded promising results, with studies reporting patients achieving multi-year circulating FVIII levels within the normal/healthy range.^{5,6} The longevity of FVIII expression after AAV delivery is unknown, but it is not expected to be lifelong. A cellular-based FVIII

delivery product could overcome the limited therapeutic expression window as well as known risks associated with *in vivo* gene delivery, such as the possibility of off-target transduction⁷ and transient hepatotoxicity induced by viral capsids,⁸ which can trigger subsequent immune/inflammatory destruction of many of the transduced cells.⁹ In addition, a gene delivery approach utilizing cells modified *ex vivo* to express FVIII could be used to treat patients who have pre-existing,^{10,11} or who develop, neutralizing antibodies to AAV.

The realization of the full potential of a cell-based gene delivery requires the identification and use of optimal FVIII constructs that are able to supply a FVIII molecule (1) that can be produced by the cell without inducing cellular stress responses,¹² (2) that exhibits enhanced functionality, and (3) that is secreted at therapeutic levels. In addition to an optimized transgene, the gene-modified cells have to be able to efficiently produce and secrete FVIII, and they should lodge/engraft and persist for the long term within a broad range of tissues upon infusion, in the absence of conditioning. Thus, these cells have to be relatively immune-inert to evade the immune system, even when expressing therapeutic proteins that the recipient perceives as foreign.

We recently tested the therapeutic potential of FVIII-expressing bone marrow-derived mesenchymal stromal cells (MSCs) in a line of sheep that emulates the genetics, inhibitor formation (to administered FVIII protein), and clinical symptoms of the severe form of human HA.¹³ We showed that the postnatal intraperitoneal (i.p.) transplantation of haploidentical MSCs engineered to express expression/secretion-optimized B domain-deleted porcine FVIII led to complete

Received 19 February 2020; accepted 9 March 2020;
<https://doi.org/10.1016/j.omtm.2020.03.001>.

⁶These authors contributed equally to this work.

⁷Senior author

Correspondence: Graça Almeida-Porada, Wake Forest Institute for Regenerative Medicine, Fetal Research and Therapy Program, Wake Forest School of Medicine, 391 Technology Way, Winston-Salem, NC 27157-1083, USA.

E-mail: galmeida@wakehealth.edu



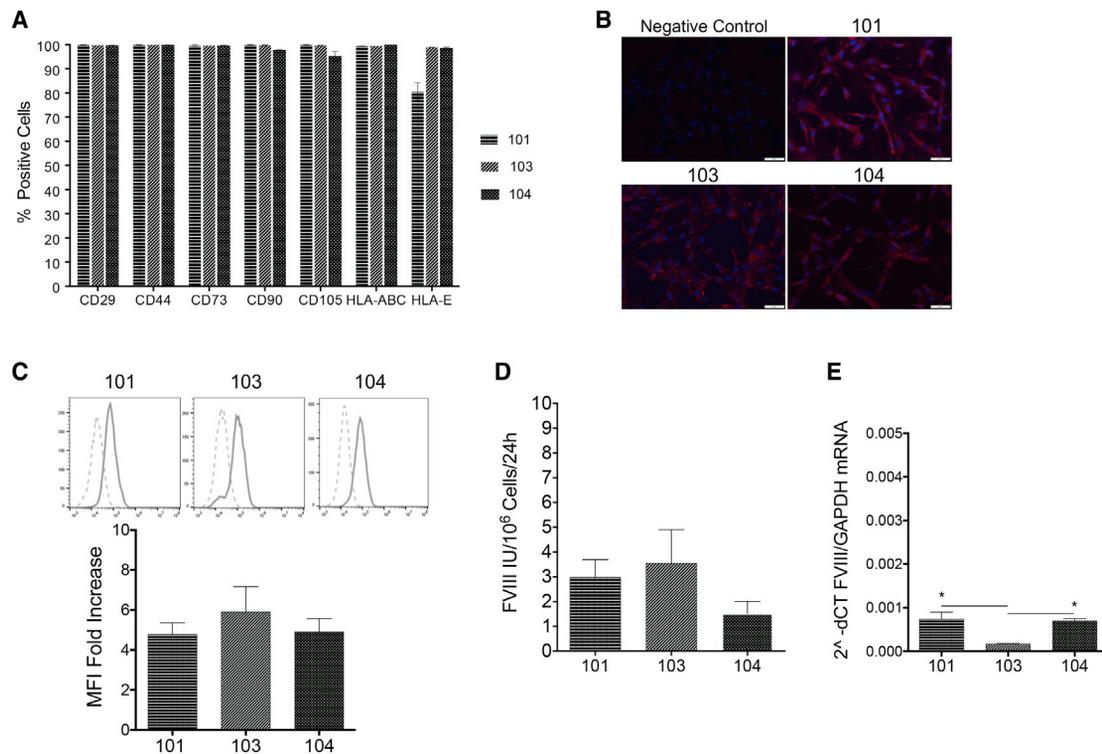


Figure 1. Characterization of Phenotype and FVIII Production by Placental Cell Master Banks

(A) Flow cytometric characterization of cultured PLC101, PLC103, and PLC104 demonstrating the expression of characteristic mesenchymal markers ($n = 3$). (B) Representative image (original magnification, $\times 40$) of immunofluorescence evaluation of FVIII expression by PLCs using an antibody specific for FVIII, showing that these cells constitutively produce FVIII protein (in red); DAPI (in blue) labels all nuclei ($n = 5$); the negative control consisted of slides stained in parallel, in which the primary antibody was absent. (C) Flow cytometric analysis of FVIII expression by PLCs. Upper panel: Solid-line histograms show fluorescence data for FVIII, and the dashed-line histograms depict the respective isotype controls. Lower panel: The median fluorescence intensity (MFI) ratio was obtained by dividing the MFI of FVIII by the respective MFI of the isotype ($n = 3$). (D) Evaluation by aPTT of FVIII activity in 24-h culture supernatants harvested from PLCs, showing the amount of functional FVIII in IU being produced by 10^6 cells ($n = 5$). (E) Endogenous levels of FVIII mRNA determined by qRT-PCR, after normalization to respective GAPDH levels, in PLC101, PLC103, and PLC104 ($n = 3$). Experimental results are presented as the mean \pm the standard error of the mean. * $p < 0.05$ was considered statistically significant.

phenotypic correction of two pediatric HA sheep, reversal of existing hemarthroses, and return to normal physical activity.¹⁴ Remarkably, this phenotypic correction was long-lasting despite the presence of high-titer inhibitors in these sheep, and the engrafted MSCs were not cleared by the recipient's immune system, enabling them to persist long-term in multiple sites, expressing FVIII. However, we found that despite the high level of transduction ($>95\%$),¹⁴ bone marrow-derived MSCs, on average, produced only 0.83 IU of FVIII/24 h/ 10^6 cells, leading us to investigate the suitability of other cells and FVIII transgenes as delivery platforms for treating HA. Similar to MSCs,^{14–20} human placenta-derived mesenchymal cells (PLCs) possess a set of several fairly unique properties that make them ideal both for cellular therapies/regenerative medicine²¹ and as vehicles for gene and drug delivery,^{22,23} as they can be easily isolated from full-term pregnancies, extensively expanded in culture, and successfully banked for clinical applications.²³

In this study, we compared three different banked PLC master cell banks for their ability to serve as vehicles for FVIII delivery following

lentiviral vector (LV) transduction, and we investigated whether this gene modification resulted in altered function, phenotype, or expression of immune markers or stress molecules by PLCs. In addition, since the pharmaceutical properties of FVIII can be markedly enhanced by codon optimizing the nucleotide sequence for the intended target cell or tissue and by including amino acid substitutions known to facilitate endoplasmic reticulum (ER) processing and secretion,^{24–27} we also performed a head-to-head comparison to identify the FVIII transgene sequence that yielded optimal FVIII expression and secretion from PLCs.

RESULTS

Characterization of PLCs

In order to investigate the suitability of fetal-derived term PLCs as cellular delivery vehicles for FVIII, we first determined the phenotypic profile of PLCs derived from three different donors/master cell banks (PLC101, PLC103, and PLC104). PLCs were isolated as described in detail in the [Materials and Methods](#), and the results of this phenotypic analysis are shown in [Figure 1](#). No statistically

significant differences ($p > 0.05$) in phenotype were found between PLCs derived from the different master cell banks, as determined by flow cytometric analysis. PLCs expressed CD29, CD44, CD73, CD90, CD105, human leukocyte antigen (HLA)-ABC, and HLA-E (Figure 1A) and exhibited negligible amounts (<1%) of CD31, CD34, CD45, CD144, HLA-G, and HLA-DR/DP/DQ, and they were devoid of A and B blood group expression, as determined by immunofluorescence microscopy (Figure S1A). PLCs constitutively expressed readily detectable levels of FVIII protein, as determined by immunofluorescence microscopy (Figure 1B) and by flow cytometry (Figure 1C) using antibodies specific for FVIII:C. In addition, PLCs secreted functional FVIII as determined by an activated partial thromboplastin time (aPTT)-based one-stage coagulation assay performed on 24-h culture supernatants harvested from cells plated at the same density. Figure 1D depicts PLC levels of FVIII secretion after normalization for the number of cells present at the time of supernatant collection, as described in Materials and Methods. No significant differences were found between the three PLC master cell banks regarding endogenous production of FVIII protein ($p > 0.05$).

Quantitative reverse transcriptase PCR (qRT-PCR) was used to evaluate the endogenous FVIII mRNA levels in the different PLCs. PLC101, PLC103, and PLC104 all had detectable FVIII mRNA. The level of FVIII mRNA in each cell population was calculated as a normalized individual data point, by comparing the threshold cycle (Ct) value for FVIII with the Ct of the respective internal reference gene GAPDH²⁸ using the formula $2^{-\Delta\Delta Ct}$. No significant difference ($p > 0.05$) in endogenous FVIII mRNA expression was observed between PLC101 and PLC104, but, unexpectedly, PLC103 had lower FVIII mRNA levels when compared to the other two PLC populations ($p < 0.05$) (Figure 1E).

As PLCs were derived from donors who were serologically positive for cytomegalovirus (CMV), we performed quantitative PCR (qPCR) with primers specific for the US6 HCMV gene as previously described²⁹ and detailed in the Supplemental Materials and Methods. No amplification of US6 CMV occurred in DNA isolated from PLC101, PLC103, or PLC104, while DNA extracted from bone marrow-derived mononuclear cells from a known CMV-positive donor was readily amplified for US6 CMV under the same conditions (data not shown).

Evaluation of Transduction Efficiency, FVIII Production, and FVIII Secretion of Three Different PLC/Master Cell Banks

In order to maximize the therapeutic benefit that could be provided by administering these cells in the context of severe HA, we next assessed the suitability of PLCs as a transgenic FVIII production platform. First, we determined whether PLCs expressed LDL-R, as this receptor has been described as the major entry port of vesicular stomatitis virus G protein (VSV-G)-pseudotyped LV in human cells.³⁰ All three master cell lines uniformly expressed LDL-R (>99% \pm 0.04%) ($n = 3$) (Figure S1B). Next, PLC101, PLC103, and PLC104 were transduced at an MOI of 7.5 using mcoET3-LV, a VSV-G-pseudotyped LV encoding a myeloid-codon-optimized (mco), bioengineered FVIII

transgene that contains high-expression elements from porcine FVIII (ET3), as detailed in Materials and Methods. After transduction, the production of FVIII was once more confirmed using immunofluorescence microscopy (Figure 2A), and secretion was determined by aPTT performed on 24-h culture supernatants harvested from PLCs plated at the same density and normalized for the number of cells present at the time of supernatant collection. Levels of FVIII in the culture supernatant increased significantly ($p < 0.05$) in all three transduced PLCs when compared with respective non-transduced PLC counterparts (Figure 2B). In addition, PLC103 ($p < 0.05$) and PLC104 both produced higher levels of FVIII when compared with PLC101, but the differences in FVIII secretion between transduced master cell banks 103 and 104 were not statistically significant (Figure 2B), even after normalization for vector copy number (Figure S2A).

To confirm that the FVIII being produced/secreted by the transduced PLCs was comparable to native FVIII, we performed enzyme-linked immunosorbent assay (ELISA) studies to quantify the concentration of FVIII protein present in the supernatant of each transduced PLC population, and then used the data from the aPTT assays on these same supernatants to calculate the specific activity (IU/mg) of the protein product of each FVIII transgene when secreted by human PLCs. These studies revealed comparable specific activities for all transgene products that were in the range of that reported for fully activated human FVIII (~100,000 IU/mg), suggesting that the majority of the FVIII being secreted by the PLCs was activated (FVIIIa). To address this issue directly, we performed activation quotient (AQ) assays using both one-stage and two-stage coagulation assays as described previously,³¹ defining the activation quotient as the ratio of FVIII activity measured by a two-stage coagulation assay divided by the FVIII activity measured by the one-stage coagulation assay. Typically, the AQ value for purified recombinant FVIII that is devoid of FVIIIa is >20 and <80. These assays revealed AQ values of roughly 1 for all samples, again supporting the conclusion that the FVIII being released by the PLCs was largely present as FVIIIa, thus suggesting that enzymes present on the surface of the PLCs and/or serum components within the PLC media were activating the vector-encoded FVIII upon release.

In order to determine the fold increase in the levels of FVIII mRNA following transduction with mcoET3-LV, levels for the mcoET3 mRNA transgene were determined in mcoET3-LV-transduced master cell banks by qRT-PCR, using primers specific to a region of mcoET3 that differs from the endogenous FVIII sequence, and compared to the basal levels of endogenous FVIII mRNA present in the respective non-transduced control, using primers specific to the B domain, which is absent in mcoET3 ($n = 3$). As shown in Figure 2C, mcoET3 mRNA levels in transduced PLC101, PLC103, and PLC104 were all significantly higher than the levels of endogenous FVIII mRNA in the corresponding non-transduced PLCs, after normalization to the respective GAPDH values. In addition, comparison of the three transduced cell banks demonstrated that PLC103 had significantly higher relative levels of mcoET3 than did PLC101

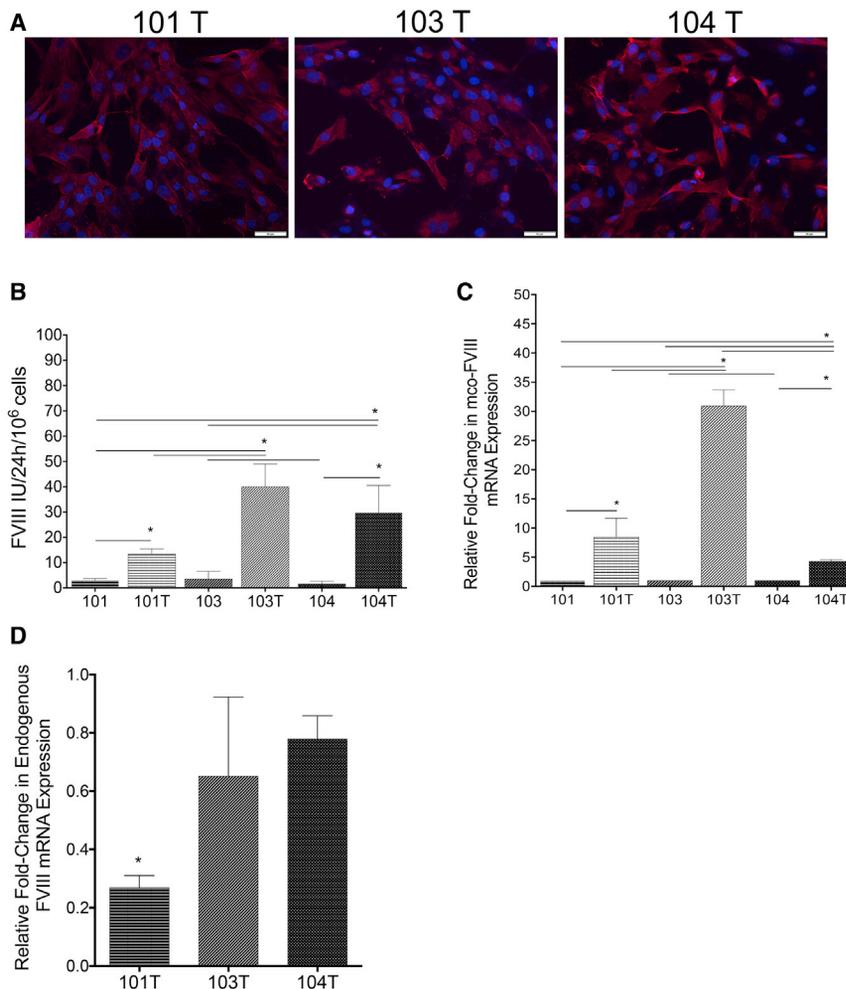


Figure 2. Evaluation of FVIII Production and FVIII Secretion of Three Different PLCs/Master Cell Banks by Immunofluorescence Microscopy, aPTT, and qPCR after Transduction with mcoET3-LV

(A) Representative images (original magnification, $\times 40$) of immunofluorescence analysis of mcoET3-LV-transduced PLC101, PLC103, and PLC104, at the same MOI of 7.5, using an antibody specific for FVIII (red); DAPI (in blue) labels all nuclei ($n = 5$). (B) Amount of functional FVIII (IU/ 10^6 cells), evaluated by aPTT, present in 24-h culture supernatants harvested from PLCs that were plated at the same density and normalized for the number of cells present at the time of supernatant collection ($n = 5$). (C) mRNA levels for the mcoET3 transgene were determined by qRT-PCR in all mcoET3-LV-transduced master cell banks, using primers specific for a region of mcoET3 that differs from the endogenous FVIII sequence, and compared to the levels of endogenous FVIII in the respective non-transduced cells, using primers specific for the FVIII B domain, after normalization of each sample's value to its respective GAPDH control ($n = 3$). (D) Relative fold change in endogenous FVIII mRNA after transduction with mcoET3-LV in PLC101, PLC103, and PLC104 ($n = 3$). Experimental results are presented as the mean \pm the standard error of the mean. * $p < 0.05$ was considered statistically significant.

or PLC104 (Figure 2C), even after normalization for vector copy number (Figure S2B).

To establish whether expression of mcoET3 affected steady-state endogenous FVIII mRNA levels, qRT-PCR was used to compare the relative FVIII mRNA levels in transduced and non-transduced PLCs ($n = 3$), again using primers specific to the B domain only present in endogenous FVIII mRNA. Although all transduced cells decreased their relative endogenous FVIII mRNA after transduction, only transduced PLC101 cells were found to have a significant decrease ($p < 0.05$) in relative endogenous FVIII mRNA, when compared with their untransduced counterparts (Figure 2D).

Transduction of PLCs with LV Vector Encoding FVIII Does Not Change Function or Phenotype, and It Does Not Upregulate Cell Surface Immune Markers or Stress Molecules

Population doubling times (PDTs) were compared between transduced (PDT = 11.3 ± 0.3 h) and non-transduced PLCs (PDT = 14.2 ± 1.8), and results demonstrated that transduction did not exert a statistically significant effect on cell growth. Next, we determined

whether transduction of PLCs with mcoET3-LV affected the phenotype (Figure 3A) or expression of signaling molecules involved in immunity (Figures 3B and 3C). PLCs transduced with mcoET3-LV continued to express CD29, CD44, CD73, CD90, CD105, CD155, and CD47, with no statistically significant differences found between transduced and non-transduced cells ($p > 0.05$). Of note is that the percentage of cells expressing CD58 differed between master cell banks, with a higher percentage of PLC103 expressing this molecule ($p < 0.05$) (Figure 3B). However, transduction did not significantly affect the frequency of CD58-expressing cells, as the percentage of CD58⁺ cells in each cell bank remained similar before and after transduction. A significantly higher percentage of non-transduced PLC103 ($85.05\% \pm 5.55\%$; $p < 0.05$) expressed CD112³² when compared to the other two PLCs. After transduction, however, the frequency of CD112-expressing cells was similar in all three transduced cell banks (Figure 3B), since the percentage of PLC103 and PLC101 positive for CD112 decreased to equal that of PLC104. HLA class II molecules HLA-DR/DP/DQ were expressed in fewer than 5% of non-transduced PLCs, and transduction did not significantly alter this expression pattern ($p > 0.05$) (Figure 3C). HLA-E, a member of the nonpolymorphic HLA class Ib molecules, which plays an important role in fetal-maternal tolerance and as a mediator of the innate and adaptive immune response, was expressed by PLCs to varying degrees, with a higher percentage of PLC103 and PLC104 expressing HLA-E than did PLC101 ($p < 0.05$) (Figure 3C). After transduction, the percentage of PLC103 and

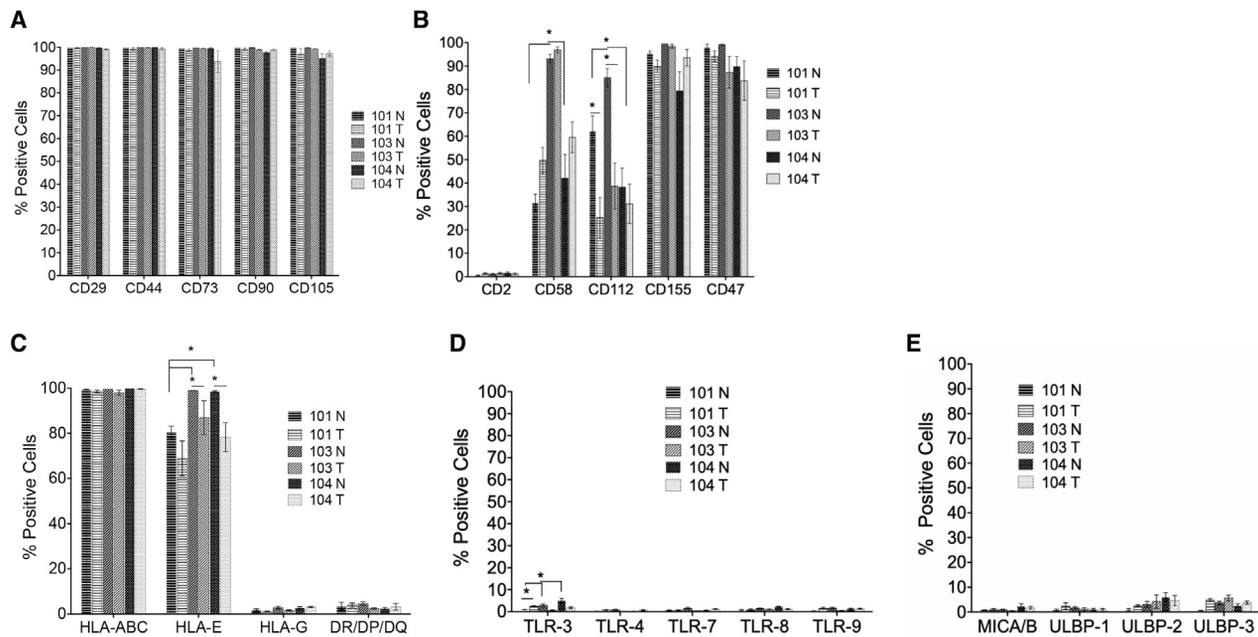


Figure 3. Phenotype of Non-transduced and mcoET3-LV-Transduced PLCs

(A) Flow cytometric analysis of non-transduced and mcoET3-LV-transduced PLCs demonstrated that the mesenchymal phenotype of PLCs was maintained following transduction ($n = 3$). (B) Flow cytometric analysis of non-transduced and transduced PLC banks for immune receptors/markers ($n = 3$). (C) HLA-I and HLA-II expression on transduced and non-transduced PLCs evaluated by flow cytometry ($n = 3$). (D and E) Flow cytometric evaluation of Toll-like receptor molecules (D) and stress molecules (E) in transduced and non-transduced PLCs ($n = 3$). Experimental results are presented as the mean \pm the standard error of the mean. * $p < 0.05$ was considered statistically significant.

PLC104 that were positive for HLA-E significantly decreased (Figure 3C) when compared to corresponding untransduced PLCs ($p < 0.05$). Of note is that the percentage of transduced PLC103 expressing HLA-E was still higher ($p < 0.05$) than that observed in untransduced PLC101.

We also investigated whether transduction of PLCs with mcoET3-LV induced upregulation of Toll-like receptor (TLR) molecules. The overall percentage of PLCs expressing the various TLRs was very low ($<5\%$; Figure 3D), and transduction produced no significant alteration in the expression of TLRs 3, 4, 7, 8, or 9 in PLC103 and PLC104, as determined by flow cytometric analysis (Figure 3D). Although PLC101 increased TLR3 and TLR4 expression after transduction, still less than 3% and 1% of the cells, respectively, expressed these markers.

Given the demands that expressing FVIII can place on the ER and secretory pathway,^{12,33–35} we also investigated whether PLC transduction induced cellular stress. Since unfolded protein response (UPR) pathways during ER stress results in upregulation of cell surface proteins such as ULBP and downregulation of MICA/MICB (MICA/B),³⁶ which could trigger an immune response to the transplanted PLCs, we quantified changes in expression of MICA/B and ULBP1, ULBP2, and ULBP3 on transduced cells.

Flow cytometric analysis demonstrated that MICA/B and ULBP1, ULBP2, and ULBP3 were expressed by only a small percentage of

PLC101, PLC103, or PLC104 prior to transduction, and transduction with mcoET3-LV did not significantly alter the expression of any of these stress-associated molecules ($p > 0.05$) (Figure 3E).

Since placental tissues have been reported to produce interferon γ (INF- γ)³⁷ upon stress, we also used a high-sensitivity ELISA (assay range, 0.16–10.0 pg/mL) to test the culture supernatants of transduced and non-transduced PLCs. No INF- γ was detected in the culture supernatants of these cells before or after transduction ($n = 3$; data not shown).

Evaluation of Transduction Efficiency, FVIII Production, and FVIII Secretion from PLCs following Transduction with Lentiviral Vectors Encoding Five Different FVIII Transgenes

Bioengineering FVIII through codon optimization can lead to increased synthesis, secretion efficiency, and overall functional activity.^{25,38} Therefore, in order to determine an optimized FVIII transgene to produce/secrete FVIII from PLCs, we compared mcoET3 with the non-codon-optimized ET3,²⁷ a liver codon-optimized ET3 (lcoET3), and two bioengineered human (HSQ) transgenes, that is, a myeloid codon-optimized human FVIII (mcoHSQ) and a liver codon-optimized human FVIII (lcoHSQ).^{24,25}

Since PLC103 and PLC104 displayed similar patterns of secretion after mcoET3-LV transduction, we used the PLC103 cell bank for these studies. PLC103 cells were transduced using an identical LV encoding

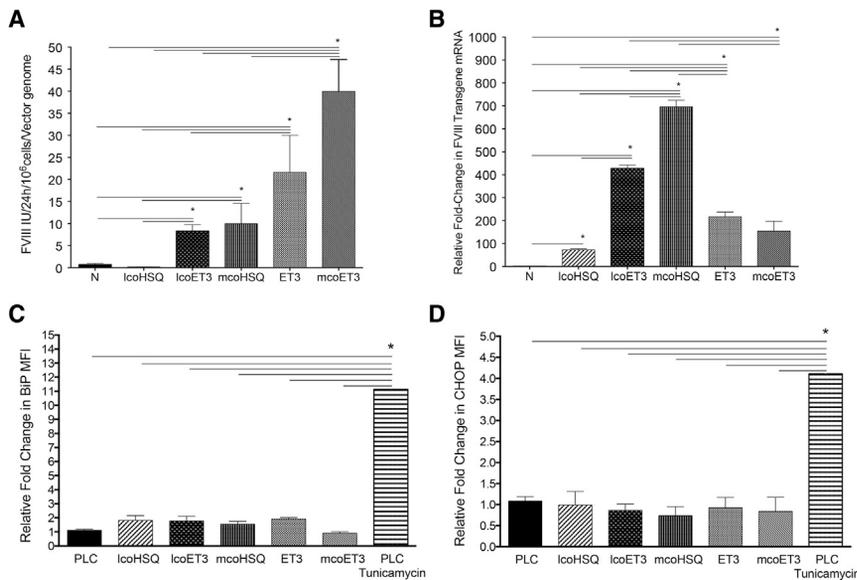


Figure 4. Determination of the Optimal FVIII Transgene for Increased Synthesis and Secretion of FVIII by Transduced PLCs

(A) Evaluation of FVIII:C activity in 24-h culture supernatants measured by aPTT ($n = 5$) after normalization for the number of cells and VCN in PLCs transduced with LVs encoding each of five different bioengineered FVIII transgenes at an identical MOI of 7.5. The transgenes used were as follows: ET3, non-codon-optimized bioengineered human/porcine hybrid FVIII; mcoET3, myeloid codon-optimized ET3; lcoET3, liver codon-optimized ET3; mcoHSQ, myeloid codon-optimized human FVIII; and lcoHSQ, liver codon-optimized human FVIII. Transduced cells were passaged three times before analysis was performed. (B) After supernatant collection, transduced PLCs were harvested and transgene-specific FVIII mRNAs were quantitated by qRT-PCR. All values were normalized to GAPDH and VCN ($n = 3$). (C and D) Flow cytometric analysis of BiP (C) and CHOP (D) in PLCs alone, PLCs transduced with the different FVIII transgenes, and PLCs treated with tunicamycin (positive control). The fold change in MFI of BiP ($n = 3$) and CHOP ($n = 3$) was calculated by

dividing the MFI of BiP or CHOP in transduced or tunicamycin-treated PLCs by the MFI of non-transduced PLCs, after subtracting the respective isotype controls. Experimental results are presented as the mean \pm the standard error of the mean. * $p < 0.05$ was considered statistically significant.

each of the five different transgenes at the same MOI of 7.5. Transduced cells were passaged three times before analysis was performed. In order to resolve any potential differences in the levels of transduction by the various vectors, we also established the gene transfer efficiency by determining final proviral copy number (vector copy number [VCN]) using qPCR. The VCNs of ET3-PLCs, mcoET3-PLCs, lcoET3-PLCs, lcoHSQ-PLCs, and mcoHSQ differed, being 0.8 ± 0.4 , 0.5 ± 0.09 , 0.81 ± 0.4 , 1.2 ± 0.4 , and 0.5 ± 0.05 , respectively. Results of evaluation of FVIII:C activity in 24-h culture supernatants after normalization for the number of cells and VCNs are displayed in Figure 4A. PLCs transduced with LV encoding mcoET3 (mcoET3-PLCs) secreted significantly higher levels of FVIII than did mcoHSQ-PLCs, lcoHSQ-PLCs, lcoET3-PLCs, and non-transduced controls ($p < 0.05$). Of note, the lcoHSQ transgene resulted in the lowest levels of secreted FVIII in PLCs of any of the FVIII transgenes tested ($p < 0.05$).

qRT-PCR was used to evaluate mRNA levels in the different transduced cells using primers designed to specifically amplify each one of the FVIII transgenes. The results, after normalization to GAPDH and VCN for each of the transduced PLCs, are shown in Figure 4B. Interestingly, despite being the cells with the highest secretion of FVIII, mcoET3-PLCs were found to have significantly lower relative mcoET3 mRNA levels than did the other transgenes, with the exception of ET3-PLCs.

No evidence of induction of UPR-related surface molecules in PLCs after transduction was found, as demonstrated by the lack of upregulation of ULBP proteins and downregulation of MICA/B. Nonetheless, we investigated whether transduction of PLCs with any of the FVIII transgenes led to upregulation of the UPR sentinel chaperone

BiP (binding immunoglobulin protein), since its synthesis was reported to be significantly induced by accumulation of misfolded FVIII proteins in the ER.^{12,33–35} In addition, we also examined whether the downstream signaling, proapoptotic protein C/EBP homologous protein (CHOP) was upregulated. The results of flow cytometric analysis of BiP and CHOP in PLCs alone, PLCs transduced with the different FVIII transgenes, and PLCs treated with tunicamycin (positive control), as described in the Supplemental Materials and Methods, are shown in Figure 4C. No significant upregulation of BiP or CHOP was seen in PLCs overexpressing FVIII when compared to PLCs alone, as determined by calculating the fold change in mean fluorescence intensity (MFI) of BiP and CHOP, after subtracting respective isotype controls.

Integration Site Analysis of PLCs Transduced with the Optimal FVIII Transgene to Produce/Secrete FVIII

Product safety is paramount for the translation of these studies, and thus we performed integration site analysis on mcoET3-PLCs, since this was the cell and transgene combination found to produce/secret the optimal amounts of FVIII. We unequivocally identified 24 unique integration sites on chromosomes 1, 2, 3, 5, 7, 9, 10, 11, 12, 13, 14, 16, 17, 19, and 21, as depicted in Figure 5. Of these 24 integration sites, 19 were located in the body of protein-coding genes (79%), while 5 (21%) were located in intergenic sequences. No preference for integration near transcription start sites was found, which agrees with what has been reported in other studies using LVs.^{39–41} Importantly, none of these integration events occurred within, or in close proximity to, known oncogenes or tumor repressors. Moreover, the continued presence of multiple distinct integration sites despite repeated passaging strongly suggests that clonal dominance did not take place.

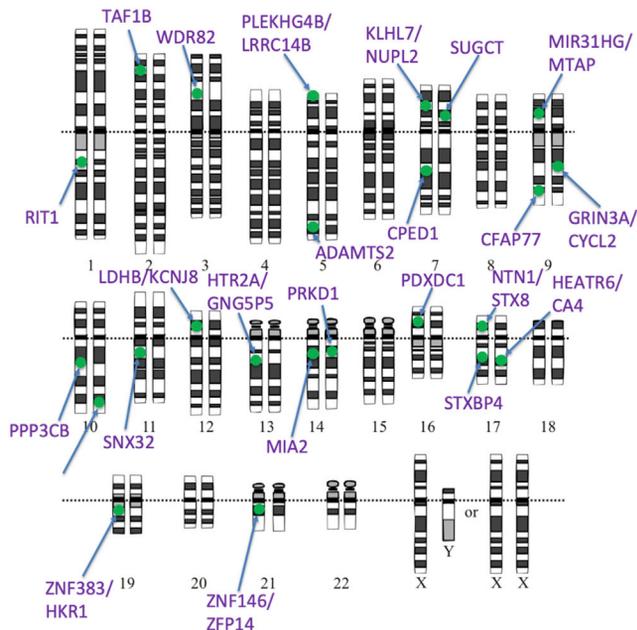


Figure 5. Integration Site Analysis of PLCs Transduced with the Optimal FVIII Transgene to Produce/Secrete FVIII

24 unique integration sites on chromosomes 1, 2, 3, 5, 7, 9, 10, 11, 12, 13, 14, 16, 17, 19, and 21 of transduced PLCs were identified. Their chromosomal locations are graphically depicted with green dots in the left panel. The purple text indicates the name of the gene closest to the insertion site.

DISCUSSION

The availability of FVIII protein replacement therapy, including the recently introduced long half-life products, revolutionized the care of HA patients, restoring a nearly normal life expectancy and greatly improving quality of life.¹ However, these products (1) are not available to the vast majority of the world's HA patients, (2) are prohibitively expensive, and (3) require lifelong therapy.⁴² As a result, the delivery of FVIII through gene and/or cellular therapy has long been recognized as a promising approach for providing long-term correction and, perhaps, permanent cure for HA.^{2,4,25,43–47} While recent clinical trials involving the direct injection of AAV-based vectors encoding FVIII have reported patients achieving sufficient circulating FVIII:C levels to yield phenotypic correction,^{48,49} it is not yet clear whether this approach will result in permanent cure, and the direct injection of viral-based vectors carries with it the inherent risks of off-target transduction and of induction of an inflammatory and/or immune response to the infused vector which, at best, requires immunosuppression and, at worst, results in toxicity.⁴⁹

Some murine studies demonstrated that AAV is able to integrate into the genome of hepatocytes and cause hepatocellular carcinoma,⁵⁰ and a recent report documented significant levels of integration of AAV vectors in hepatocytes of non-human primates after prenatal administration,⁵¹ raising additional concerns. However, thus far these adverse effects have not been reported in any of the clinical trials using AAV.

The use of cells as a vehicle to deliver a FVIII transgene has several inherent advantages over the direct injection of the viral vector. First, this approach eliminates the possibility of off-target transduction, as well as an inflammatory/immune response to the vector itself. In addition, the cells that are transduced can be extensively characterized prior to infusion for expression of the transgene product (i.e., potency), and genomic analyses can also be performed to verify the safety and stability of the cells. Such analyses are particularly important when integrating vectors are used with the goal of achieving life-long correction following a single intervention. For instance, prenatal treatment (PNTx) for HA^{42,52} could induce lifelong tolerance to FVIII and thereby eliminate the risk of inhibitor formation, the most feared problem in treatment/management of HA. Although direct injection of viral vectors into prenatal recipients would likely be successful, numerous safety concerns need to be addressed before the direct injection of viral vectors into prenatal recipients is clinically possible. A cell and gene therapy-based approach, in which cells are transduced *in vitro*, would enable multiple safeguards to be implemented in production that are not possible with direct vector injection.

Herein, we performed studies with the overall goal of developing an optimized cellular platform for delivering a FVIII transgene to treat HA. This treatment could be used not only in prenatal recipients, but also in those patients who, during their lifetime, develop FVIII inhibitors. Indeed, we have previously shown the feasibility of overcoming bleeding and reversing existing hemarthroses in animals with high-titer inhibitors after transplantation of bone marrow-derived haploidentical MSCs engineered to overexpress FVIII.¹⁴ These studies also demonstrated that, upon administration, modified MSCs were not cleared by the recipient's immune system, enabling them to persist long-term in multiple sites, attesting to the feasibility of a cell therapy to overcome inhibitors.¹⁴ Despite this promising outcome, however, the levels of FVIII produced and secreted by gene-modified bone marrow-derived MSCs were low. We therefore reasoned that a cell that endogenously produces and secretes high levels of functional FVIII would be ideal as a delivery vehicle, as such a cell would possess all of the necessary machinery to efficiently produce and secrete vector-driven FVIII while maintaining viability and normal function. Combining such a cell with a FVIII transgene, such as ET3, that has been optimized for high-level expression and secretion without altered immunogenicity,² would enable the transplantation of a smaller cell dose/kg to achieve the desired therapeutic effect, improving the safety of this treatment approach. The current study demonstrates that human MSCs isolated from the fetal side of the full-term placenta endogenously synthesize mRNA for FVIII, translate this mRNA into protein, and that they are able to efficiently process and secrete functional FVIII protein that has normal procoagulant activity when measured using a standard clinical aPTT assay. In addition to their ability to produce and secrete functional FVIII, human PLCs exhibit a repertoire of surface markers that is consistent with that of MSCs isolated from the bone marrow and other tissues.⁵³ They express very low/undetectable levels of HLA class II and are devoid of expression of blood group antigens A and B. As such, PLCs, similar to MSCs from other sources, are likely to be relatively

immune-inert,²³ and they could potentially be used as an off-the-shelf cellular therapy without the need for immunosuppression. In this study, we demonstrated that human PLCs isolated from three different master cell banks were all readily transduced at rates of more than 90% with a LV encoding mcoET3, a transgene that, when compared to the native human *FVIII*, exhibits markedly enhanced production and secretion.⁵⁴ Following transduction, all three PLC lines expressed significantly higher levels of mRNA (5- to 30-fold, depending on the PLC line) and secreted markedly enhanced levels of functionally active FVIII into the culture supernatant (5- to 20-fold above basal levels, depending on the PLC line), firmly establishing the suitability of PLCs as cellular vehicles for delivering a *FVIII* transgene, especially PLC103, which exhibited the highest levels of FVIII protein and procoagulant activity following transduction.

Interestingly, the levels of mRNA for endogenous FVIII were found to be affected by transduction with specific FVIII transgenes. Although we do not have a definitive explanation for this observation, two unintentional consequences of the optimization process employed to achieve maximal FVIII expression and secretion may provide insight into the responsible mechanisms. First, as described,^{55,56} the native FVIII mRNA contains a sequence that acts to suppress its accumulation within the cytoplasm and a region that has been reported to contain an autonomously replicating sequence that binds to a nuclear factor to repress transcription of the FVIII gene. Introducing porcine elements into the human FVIII sequence to enhance secretion and performing codon optimization altered the degree of homology that each our FVIII transgenes possessed to these repressor regions, which could well have impacted the transcription, accumulation, and/or stability of the resultant transgene's mRNA and could also have resulted in "bystander effects" on the transcription and stability of the endogenous FVIII mRNA within the cell. Second, the process of codon optimization also resulted in the introduction of new binding sites for microRNAs (miRNAs) that have been reported to be expressed within the placenta and by MSCs from various tissues.⁵⁷⁻⁶¹ Although beyond the scope of the present study, it is plausible that these newly introduced miRNA binding sites could have negatively affected the stability/half-life of the mRNA for the respective FVIII transgenes, and that a thorough investigation into this aspect of FVIII mRNA stability could yield further gains in transgene expression.

Equally importantly, transduction with the mcoET3-LV did not alter the phenotype, viability, or function of the PLCs, nor did it significantly alter the expression of molecules associated with innate immunity. Since lentiviruses possess a double-stranded RNA genome, we examined TLR3, as this molecule is triggered by the presence of double-stranded RNA. The percentage of cells expressing TLR3 was less than 5%, irrespective of the cell bank or transduction. Although the increase we observed from 1% TLR3⁺ cells to approximately 2% TLR3⁺ cells in PLC101 following transduction achieved statistical significance, we do not think that this small change is likely to be of biological significance. Similarly, although the percentage of CD58-expressing cells differed between PLC banks, the percentage of cells

expressing this molecule was not affected by transduction. Of note is that interaction of CD58 molecules on the PLCs with CD2 present on the surface of T cells does not lead to T cell activation, due to the lack of expression of T cell costimulatory molecules on PLCs,^{62,63} but instead generates a Foxp3-negative CD4/CD8⁺ regulatory T (Treg) cell population that is highly suppressive of the immune response.⁶⁴ We and others have previously reported that MSCs from bone marrow do not express appreciable levels of the immunosuppressive HLA class Ib molecule, HLA-E.^{65,66} Interestingly, all three PLC banks exhibited high constitutive expression of this molecule, with 80%–100% of cells expressing HLA-E, depending on the bank. HLA-E plays a critical role in immune surveillance by interacting with CD94/NKG2 receptors on the surface of NK cells, and this interaction can either inhibit or stimulate NK cell recognition/reactivity depending on the repertoire of peptides being presented by HLA-E.⁶⁷⁻⁷⁰ As such, it is hard to predict what effect, if any, our observation that the percentage of PLCs expressing HLA-E was reduced to some degree following transduction with an LV, especially since $\geq 80\%$ of cells from all three PLC lines still exhibited HLA-E expression following transduction. Future studies are planned to define the peptide repertoire being presented by HLA-E on the surface of the PLCs prior to and following transduction to address this issue.

The synthesis and secretion of FVIII places a great deal of stress on the host cell, as the FVIII protein is relatively large and must undergo extensive post-translational modification to function properly.^{12,33-35,71} Evaluation of BiP and CHOP in transduced cells demonstrated that no significant upregulation of these proteins occurred. Nevertheless, because UPR activation during ER stress results in upregulation of ULBP proteins and downregulation of MICA/B,³⁶ this could trigger an unwanted immune response to the transplanted cells and graft loss. We also showed that overexpression of FVIII in PLCs did not alter expression of MICA/B and ULBP1, ULBP2, and ULBP3. Overall, the inherently low immunogenicity of PLCs appears to have been preserved following LV transduction, suggesting that the use of these transduced cells as therapeutics should not trigger an inflammatory/immune response. In addition, integration site analysis further validated the safety of the gene-engineered PLCs, as none of the 22 identified integration sites was within, or in close proximity to, known oncogenes or tumor repressors. Moreover, the continued presence of multiple distinct integration sites despite repeated passaging strongly suggests that clonal dominance did not take place following transduction.

Since prior studies have shown that the levels of FVIII production following gene delivery can be dramatically enhanced by including sequence elements that enhance ER processing and decrease ER transit time, and by using computer algorithms to optimize codon usage for the particular target tissue/cell type being transduced, we also performed a direct head-to-head comparison of FVIII production/secretion following transduction of PLC103 with an identical LV encoding five different bioengineered *FVIII* transgenes, three of which were based on ET3, a hybrid human/porcine *FVIII* transgene, and two of which were based on a B domain-deleted

human *FVIII* transgene (HSQ). Interestingly, transduction of the same PLC line at the same MOI (7.5) with an identical LV encoding each of these five *FVIII* transgenes yielded markedly different levels of transduction, ranging from 14% to 95%. The reason for this difference in transduction efficiency is not clear, as the conditions for transduction were identical, the vector backbone employed was the same throughout, and the levels of LDL-R, the major entry port of VSV-G-pseudotyped LVs³⁰ in human cells, were similar between the cell lines.

Of equal note was the finding that, once adjusted for vector copy number, the level of transgene mRNA being expressed did not directly correlate with the amount of functional FVIII being secreted by the transduced PLCs. Indeed, the mRNA for mcoET3 was expressed at the second lowest level of any of the *FVIII* transgenes, yet PLCs transduced with the LV encoding mcoET3 secreted substantially higher (3- to 7-fold) levels of active FVIII than did the same PLCs transduced with an LV encoding any of the other *FVIII* transgenes. In contrast, PLCs transduced with the LV encoding mcoHSQ, the myeloid odon-optimized human *FVIII* transgene, expressed significantly higher (up to 7-fold) levels of transgene mRNA than did PLCs transduced with LV encoding any of the other *FVIII* transgenes, yet they secreted some of the lowest levels of active FVIII of any of the cell lines. Given that both mcoET3 and mcoHSQ underwent the same optimization for myeloid codon usage, these data highlight the importance of the porcine elements present in ET3²⁷ to achieve efficient production and secretion of vector-driven FVIII, irrespective of the levels of mRNA that are transcribed by the target cell. Nevertheless, our data also underscore the value in codon optimizing the *FVIII* transgene, as the ET3 and HSQ transgenes that were codon optimized for expression in the liver (lco) yielded the two lowest levels of FVIII secretion of any *FVIII* transgenes tested following transduction of PLCs. Taken together, our data suggest that maximizing FVIII expression following gene transfer will likely require both codon optimization that is specific to the desired cell type and bioengineering of the *FVIII* transgene to ensure efficient post-translational processing and secretion.

In conclusion, our studies have firmly established the utility of PLCs as cellular vehicles for delivering a *FVIII* transgene and achieving clinically meaningful levels of secreted FVIII activity. They have also demonstrated that optimizing the *FVIII* transgene for myeloid codon usage and concurrently including elements from the porcine *FVIII* sequence that are known to enhance post-translational processing markedly boosts the levels of functional FVIII that are secreted by PLCs following LV transduction. Our finding that transduction with an LV encoding this optimized *FVIII* transgene preserves the phenotype, viability/function, genomic stability, and relatively immune-inert properties of PLCs further adds to the therapeutic potential of these cells as vehicles for delivering FVIII. It is hoped that with further preclinical studies to establish *in vivo* safety and efficacy, mcoET3-LV-transduced PLCs can be moved forward as a long-lasting/curative treatment option for patients with HA.

MATERIALS AND METHODS

Isolation and Culture of Placental Cells

Human placentas were obtained from full-term deliveries after informed consent according to guidelines from the Office of Human Research Protection at Wake Forest Health Sciences. Tissue was isolated from the fetal layer within 8 mm of the chorion, thereby avoiding visible blood vessels, connective tissue, and invaginations from the maternal edge. To obtain placental stromal/stem cells (PLCs), placental tissue was minced, subjected to enzymatic digestion, and plated in placental cell growth media (PCGM) consisting of α -minimum essential medium (MEM) supplemented with 15% fetal bovine serum (FBS), 19% AmnioMAX, 1% GlutaMAX, and 2.5 μ g/mL gentamicin (Thermo Fisher Scientific, Wilmington, DE, USA). PLCs were maintained in culture in a 37°C humidified incubator containing 5% CO₂ and allowed to grow for 2–3 weeks. Cells were passaged at 70%–80% confluency using TrypLE or Accutase (Thermo Fisher Scientific, Wilmington, DE, USA) and positively selected for CD117/c-kit using c-kit selection microbeads (Miltenyi Biotec, Auburn, CA, USA) following the manufacturer's instructions. After c-kit selection, cells were plated at 3–4 $\times 10^3$ /cm² and culture expanded in PCGM under the same conditions for these studies. All PLCs were tested between passage 8 and 11 from initial isolation. PLC doubling time (DT) was calculated using the following equation:

$$DT = \text{duration} \times \log_2 / \times [\log(\text{final concentration}) - \log(\text{initial concentration})]$$

(<https://www.doubling-time.com/compute.php>).

Immunofluorescence and Flow Cytometric Analysis of Human PLCs

Flow cytometric analysis of cultured PLCs was performed according to the manufacturer's instructions and as previously described⁷² using directly conjugated antibodies against human CD2, CD29, CD31, CD34, CD44, CD45, CD58, CD73, CD31, CD47, CD90, CD112, HLA-DR/DQ/DP (all from BD Biosciences, San Jose, CA, USA); CD105, TLR7, ULBP1, ULBP3, and ULBP2/5/6 (all from R&D Systems, Minneapolis, MN, USA); CD155, HLA-G, and MICA/B (Bio-Rad, Hercules, CA, USA); CD144, HLA-ABC, and HLA-E (Thermo Fisher Scientific, Wilmington, DE, USA); TLRs 3, 4, 7, 8, and 9 and LDL-R (Abcam, Cambridge, MA, USA); FVIII:C (Affinity Biologicals, Ancaster, ON, Canada); and BiP and CHOP (Cell Signaling Technology, Danvers, MA, USA). Background fluorescence was set using non-specific isotype-matched antibodies and respective fluorochromes. Cells were analyzed using a BD Accuri C6, and data were analyzed using FlowJo software (BD Biosciences, San Jose, CA, USA).

Immunofluorescence staining for FVIII:C was performed on adherent PLCs grown in chamber slides and fixed with 4% paraformaldehyde in PBS as previously described⁷³ using mouse anti-human FVIII:C (clone RFF-VIIIC/8) (Bio-Rad, Hercules, CA, USA) and donkey anti-mouse IgG Alexa Fluor 594 (Thermo Fisher Scientific, Wilmington, DE, USA). This antibody recognizes an epitope toward

the N terminus of full-length FVIII. It also recognizes the 210-, 90-, and 40-kDa cleavage products but does not cross-react with von Willebrand factor.

Controls included slides stained in parallel, in which the primary antibody was either absent or was replaced by a non-specific isotype-matched primary antibody conjugated with Alexa Fluor 594. Cell nuclei were stained with DAPI (Thermo Fisher Scientific, Wilmington, DE, USA), and coverslips were mounted with VectaMount AQ (Vector Laboratories, Burlingame, CA, USA).

An Olympus BX63 microscope with a $\times 20$ objective was used to visualize and capture images of antibody-mediated fluorescence. Flow cytometry was performed using a BD Accuri C6 Plus (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (version 10.6.1; Tree Star, Ashland, OR).

Transduction of PLCs with Lentiviral Vectors Expressing Five Different Bioengineered FVIII Transgenes

Subconfluent cultures of PLCs were transduced overnight in QBSF60 (Quality Biologicals, Gaithersburg, MD, USA) containing 8 $\mu\text{g/mL}$ protamine sulfate (Calbiochem, San Diego, CA, USA) at an MOI of 7.5, with third generation self-inactivating LVs containing the EF-1 α promoter and encoding any of the following bioengineered human or human-porcine hybrid FVIII transgenes kindly provided by Trent Spencer and Christopher Doering:^{24,25,27,74} ET3, a chimeric human/porcine FVIII molecule that exhibits a more than 10-fold increase in secretion efficiency from human cells when compared with human B domain-deleted FVIII (HSQ) (ET3 titer = 2.5×10^7 transduction units [TU]/mL), mcoET3 (titer = 2.66×10^7 TU/mL), lcoET3 (titer = 2.46×10^6 TU/mL), mcoHSQ (titer = 1.93×10^8 TU/mL), and lcoHSQ (titer = 7.6×10^7 TU/mL). After transduction, cells were washed and media were changed to PCGM. Cells were passaged three times before any analyses were performed.

aPTT Activity Assay to Measure Functional FVIII in Cell Supernatants

PLCs at passage 3 post-transduction, or non-transduced cells at the same passage, were plated at the same density in PCGM. Media were replaced 24 h later with phenol red-free PCGM and PLCs were incubated for another 24 h. Supernatant was then collected, centrifuged at 4°C, aliquoted, and kept at -80°C . After supernatant collection, PLCs were harvested and counted, and cell the number for each sample was recorded. DNA and RNA were also extracted. These analyses enabled the data to be correlated for cell number, FVIII mRNA levels, and proviral copy number per diploid human genome (VCN) with FVIII secretion in IU/cell.

aPTT assays were performed by the Wake Forest Baptist Medical Center Special Hematology Laboratory in accordance with standard clinical procedures, using a Top 300 CTS clinical coagulometer (Instrumentation Laboratories, Bedford, MA, USA), as described in detail in the [Supplemental Materials and Methods](#).

ELISA

The assay was performed as previously described⁷⁴ and is detailed in the [Supplemental Materials and Methods](#), using the capture murine anti-human FVIII antibody 2A9 (FVIII-2A9) to the C1 domain (Green Mountain Antibodies, Burlington, VT, USA), probed with a biotin-labeled anti-human FVIII antibody clone 4A4 (FVIII-2A9) (Green Mountain Antibodies, Burlington, VT, USA), and detected using horseradish peroxidase (HRP)-streptavidin and a chromogenic substrate. The assay has a detection limit of 3.5 ng/mL.

IFN- γ ELISA

Supernatant collected from transduced and non-transduced PLCs were also collected in pyrogen/endotoxin-free tubes and tested for the presence of IFN- γ using an IFN- γ human ELISA kit (Thermo Fisher Scientific/Life Technologies, Carlsbad, CA, USA) according to manufacturer's instruction.

VCN Determination

To determine the precise proviral copy number per diploid human genome, the VCN was measured using the Lenti-X provirus quantitation kit (Takara Bio USA, Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, genomic DNA was isolated from PLCs transduced with the different FVIII transgenes, using the provided NucleoSpin tissue kit. Serial dilutions of each of the different genomic DNAs were subjected to qPCR amplification alongside dilutions of a calibrated provirus control template (PCT), which were used to generate the standard curve. Since the viral sequences in genomic DNA and the PCT are amplified with different PCR sensitivities, a correction coefficient was incorporated in order to calculate the provirus copy number from the total qPCR copy number that was found using the standard curve, in accordance with the manufacturer's instructions. After determining the cell number equivalents represented in the genomic DNA yield using the conversion factor of 6.6 pg of DNA/diploid genome, the final result was expressed in terms of provirus copies/cell.

qPCR for HCMV US6 Gene

Total DNA was extracted from the different cell populations using the DNeasy blood and tissue kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. DNA was eluted into 100 μL of nuclease-free water and quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). The presence and quantity of viral DNA in the samples was determined by amplification of the US6 HCMV gene in a qPCR reaction as previously described,²⁹ and as detailed in the [Supplemental Materials and Methods](#).

qRT-PCR for FVIII mRNA

RNA was extracted from transduced and non-transduced PLC populations using an RNeasy mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. RNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA), and RNA integrity was assessed using the Bioanalyzer RNA 6000 Nano assay and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

RNA samples were then normalized to have a concentration of 20 ng/ μ L. 10 ng of DNA-free RNA was converted into cDNA using an OmniScript RT kit (QIAGEN, Valencia, CA, USA). SYBR Green-based qPCR was conducted by using PrimeTime qPCR primer assays (Integrated DNA Technologies, Coralville, IA, USA) using primers specific for human FVIII, and for each of the different bioengineered transgenes (primer sequences appear in the [Supplemental Materials and Methods](#)). Human GAPDH served as an internal reference/housekeeping gene and was amplified using commercially available primers (catalog no. PPH00150E, QIAGEN, Valencia, CA, USA). The qPCR master mix was loaded into MicroAmp optical 96-well reaction plates and processed in the 7300 QuantStudio 3 real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Integration Site Analysis

Integration site analysis was performed using a Lenti-X integration site analysis kit (Takara Bio, Mountain View, CA) according to the manufacturer's instructions. Resultant PCR products were then cloned using the TOPO TA cloning kit (Thermo Fisher Scientific, Wilmington, DE, USA) and transformed into competent bacterial cells. Plasmid constructs were sequenced commercially (GENEWIZ, South Plainfield, NJ, USA) and analyzed for correct cloning "landmarks" to confirm the presence of legitimate integration sites. Sequences were then mapped to the human genome using BLAT (ensembl) and BLAST (NCBI).

Statistics and Data Analysis

Experimental results are presented as the mean \pm the standard error of the mean (SEM). We used GraphPad Prism 6 to perform all statistical analyses. Comparisons between experimental results were determined either by a two-tailed Student's *t* test for single comparisons or by ANOVA for data involving multiple comparisons followed by *post hoc* analysis using the Holm-Sidak method to determine individual *p* values. *p* < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtm.2020.03.001>.

AUTHOR CONTRIBUTIONS

N.E.-A., M.R., R.R., A.M., A.R., and B.T. performed experiments, data analysis, and interpretation; D.M. and A.F. provided expertise; J.S. provided reagents; C.B.D., H.T.S., and A.A. provided reagents and experimental feedback; G.A.-P., C.D.P., C.B.D., and H.T.S. approved the final version of the manuscript; and G.A.-P. and C.D.P. conceived the experimental design, supervised experiments, performed data analysis and interpretation, wrote the manuscript, and secured funding.

ACKNOWLEDGMENTS

This work was supported by NIH/ NHLBI grants HL130856, HL135853, and HL148681. We would like to thank the Wake Forest Baptist Health Special Hematology Laboratory for excellent technical support and the performance of FVIII testing, and Dr. Julie Allickson

and the Wake Forest Institute for Regenerative Medicine Clinical Center for providing the PLC cell banks. The graphical abstract was created with BioRender.

REFERENCES

- DiMichele, D.M. (2018). Navigating speed bumps on the innovation highway in hemophilia therapeutics. *HemaSphere* 2, e144.
- Doering, C.B., Denning, G., Shields, J.E., Fine, E.J., Parker, E.T., Srivastava, A., Lollar, P., and Spencer, H.T. (2018). Preclinical development of a hematopoietic stem and progenitor cell bioengineered factor VIII lentiviral vector gene therapy for hemophilia A. *Hum. Gene Ther.* 29, 1183–1201.
- Shi, Q., Wilcox, D.A., Fahs, S.A., Weiler, H., Wells, C.W., Cooley, B.C., Desai, D., Morateck, P.A., Gorski, J., and Montgomery, R.R. (2006). Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. *J. Clin. Invest.* 116, 1974–1982.
- Porada, C.D., Sanada, C., Kuo, E., Colletti, E.J., Moot, R., Doering, C., Spencer, H.T., and Almeida-Porada, G. (2010). Phenotypic correction of hemophilia A by postnatal intraperitoneal transplantation of FVIII-expressing MSC. *Blood* 116, 249.
- Rangarajan, S., Walsh, L., Lester, W., Perry, D., Madan, B., Laffan, M., Yu, H., Vettermann, C., Pierce, G.F., Wong, W.Y., and Pasi, K.J. (2017). AAV5-factor VIII gene transfer in severe hemophilia A. *N. Engl. J. Med.* 377, 2519–2530.
- George, L.A., Ragni, M.V., Samelson-Jones, B.J., Cuker, A., Runoski, A.R., Cole, G., Wright, F., Chen, Y., Hui, D.J., Wachtel, K., et al. (2017). Spk-8011: preliminary results from a phase 1/2 dose escalation trial of an investigational AAV-mediated gene therapy for hemophilia A. *Blood* 130, 604.
- High, K.A. (2003). Theodore E. Woodward Award. AAV-mediated gene transfer for hemophilia. *Trans. Am. Clin. Climatol. Assoc.* 114, 337–351.
- Doshi, B.S., and Arruda, V.R. (2018). Gene therapy for hemophilia: what does the future hold? *Ther. Adv. Hematol.* 9, 273–293.
- High, K.A. (2014). Gene therapy for hemophilia: the clot thickens. *Hum. Gene Ther.* 25, 915–922.
- Calcedo, R., Vandenberghe, L.H., Gao, G., Lin, J., and Wilson, J.M. (2009). Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J. Infect. Dis.* 199, 381–390.
- George, L.A. (2017). Hemophilia gene therapy comes of age. *Blood Adv.* 1, 2591–2599.
- Zolotukhin, I., Markusic, D.M., Palaschak, B., Hoffman, B.E., Srikanthan, M.A., and Herzog, R.W. (2016). Potential for cellular stress response to hepatic factor VIII expression from AAV vector. *Mol. Ther. Methods Clin. Dev.* 3, 16063.
- Porada, C.D., Sanada, C., Long, C.R., Wood, J.A., Desai, J., Frederick, N., Millsap, L., Bormann, C., Menges, S.L., Hanna, C., et al. (2010). Clinical and molecular characterization of a re-established line of sheep exhibiting hemophilia A. *J. Thromb. Haemost.* 8, 276–285.
- Porada, C.D., Sanada, C., Kuo, C.J., Colletti, E., Mandeville, W., Hasenau, J., Zanjani, E.D., Moot, R., Doering, C., Spencer, H.T., and Almeida-Porada, G. (2011). Phenotypic correction of hemophilia A in sheep by postnatal intraperitoneal transplantation of FVIII-expressing MSC. *Exp. Hematol.* 39, 1124–1135.e4.
- Chamberlain, J., Yamagami, T., Colletti, E., Theise, N.D., Desai, J., Frias, A., Pixley, J., Zanjani, E.D., Porada, C.D., and Almeida-Porada, G. (2007). Efficient generation of human hepatocytes by the intrahepatic delivery of clonal human mesenchymal stem cells in fetal sheep. *Hepatology* 46, 1935–1945.
- Colletti, E., El Shabrawy, D., Soland, M., Yamagami, T., Mokhtari, S., Osborne, C., Schlauch, K., Zanjani, E.D., Porada, C.D., and Almeida-Porada, G. (2013). EphB2 isolates a human marrow stromal cell subpopulation with enhanced ability to contribute to the resident intestinal cellular pool. *FASEB J.* 27, 2111–2121.
- Colletti, E.J., Airey, J.A., Liu, W., Simmons, P.J., Zanjani, E.D., Porada, C.D., and Almeida-Porada, G. (2009). Generation of tissue-specific cells from MSC does not require fusion or donor-to-host mitochondrial/membrane transfer. *Stem Cell Res. (Amst.)* 2, 125–138.
- Colletti, E.J., Almeida-Porada, G., Chamberlain, J., Zanjani, E.D., and Airey, J.A. (2006). The time course of engraftment of human mesenchymal stem cells in fetal

- heart demonstrates that Purkinje fiber aggregates derive from a single cell and not multi-cell homing. *Exp. Hematol.* 34, 926–933.
19. Yamagami, T., Sanada, C., Wiendl, H., Zanjani, E.D., Porada, C.D., and Almeida-Porada, G. (2007). Expression of HLA-G1 and G5 enables human mesenchymal stem cells to engraft at high levels across xenogeneic barriers following transplantation into immunocompetent recipients. *Blood* 110, 363.
 20. Yamagami, T., Colletti, E., Wiendl, H., Zanjani, E., Porada, C., and Almeida-Porada, G. (2008). Expression of molecules involved in fetal-maternal tolerance allows human mesenchymal stem cells to engraft at high levels across immunologic barriers. *Blood* 112, 3480.
 21. Murphy, S.V., and Atala, A. (2013). Amniotic fluid and placental membranes: unexpected sources of highly multipotent cells. *Semin. Reprod. Med.* 31, 62–68.
 22. Bonomi, A., Silini, A., Vertua, E., Signoroni, P.B., Coccè, V., Cavicchini, L., Sisto, F., Alessandri, G., Pessina, A., and Parolini, O. (2015). Human amniotic mesenchymal stromal cells (hAMSCs) as potential vehicles for drug delivery in cancer therapy: an in vitro study. *Stem Cell Res. Ther.* 6, 155.
 23. Magatti, M., Vertua, E., Cargnoni, A., Silini, A., and Parolini, O. (2018). The immunomodulatory properties of amniotic cells: the two sides of the coin. *Cell Transplant.* 27, 31–44.
 24. Zakas, P.M., Brown, H.C., Knight, K., Meeks, S.L., Spencer, H.T., Gaucher, E.A., and Doering, C.B. (2017). Enhancing the pharmaceutical properties of protein drugs by ancestral sequence reconstruction. *Nat. Biotechnol.* 35, 35–37.
 25. Brown, H.C., Zakas, P.M., George, S.N., Parker, E.T., Spencer, H.T., and Doering, C.B. (2018). Target-cell-directed bioengineering approaches for gene therapy of hemophilia A. *Mol. Ther. Methods Clin. Dev.* 9, 57–69.
 26. Doering, C.B., Denning, G., Dooriss, K., Gangadharan, B., Johnston, J.M., Kerstann, K.W., McCarty, D.A., and Spencer, H.T. (2009). Directed engineering of a high-expression chimeric transgene as a strategy for gene therapy of hemophilia A. *Mol. Ther.* 17, 1145–1154.
 27. Doering, C.B., Healey, J.F., Parker, E.T., Barrow, R.T., and Lollar, P. (2004). Identification of porcine coagulation factor VIII domains responsible for high level expression via enhanced secretion. *J. Biol. Chem.* 279, 6546–6552.
 28. Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* 3, 1101–1108.
 29. Soland, M.A., Keyes, L.R., Bayne, R., Moon, J., Porada, C.D., St Jeor, S., and Almeida-Porada, G. (2014). Perivascular stromal cells as a potential reservoir of human cytomegalovirus. *Am. J. Transplant.* 14, 820–830.
 30. Finkelshtein, D., Werman, A., Novick, D., Barak, S., and Rubinstein, M. (2013). LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* 110, 7306–7311.
 31. Doering, C., Parker, E.T., Healey, J.F., Craddock, H.N., Barrow, R.T., and Lollar, P. (2002). Expression and characterization of recombinant murine factor VIII. *Thromb. Haemost.* 88, 450–458.
 32. Niehage, C., Steenblock, C., Pursche, T., Bornhäuser, M., Corbeil, D., and Hoflack, B. (2011). The cell surface proteome of human mesenchymal stromal cells. *PLoS ONE* 6, e20399.
 33. Lange, A.M., Altynova, E.S., Nguyen, G.N., and Sabatino, D.E. (2016). Overexpression of factor VIII after AAV delivery is transiently associated with cellular stress in hemophilia A mice. *Mol. Ther. Methods Clin. Dev.* 3, 16064.
 34. Brown, H.C., Gangadharan, B., and Doering, C.B. (2011). Enhanced biosynthesis of coagulation factor VIII through diminished engagement of the unfolded protein response. *J. Biol. Chem.* 286, 24451–24457.
 35. Malhotra, J.D., Miao, H., Zhang, K., Wolfson, A., Pennathur, S., Pipe, S.W., and Kaufman, R.J. (2008). Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc. Natl. Acad. Sci. USA* 105, 18525–18530.
 36. Hosomi, S., Grootjans, J., Huang, Y.H., Kaser, A., and Blumberg, R.S. (2018). New insights into the regulation of natural-killer group 2 member D (NKG2D) and NKG2D-ligands: endoplasmic reticulum stress and CEA-related cell adhesion molecule 1. *Front. Immunol.* 9, 1324.
 37. Banerjee, S., Smallwood, A., Moorhead, J., Chambers, A.E., Papageorgiou, A., Campbell, S., and Nicolaidis, K. (2005). Placental expression of interferon- γ (IFN- γ) and its receptor IFN- γ R2 fail to switch from early hypoxic to late normotensive development in preclampsia. *J. Clin. Endocrinol. Metab.* 90, 944–952.
 38. Dooriss, K.L., Denning, G., Gangadharan, B., Javazon, E.H., McCarty, D.A., Spencer, H.T., and Doering, C.B. (2009). Comparison of factor VIII transgenes bioengineered for improved expression in gene therapy of hemophilia A. *Hum. Gene Ther.* 20, 465–478.
 39. Wu, X., Li, Y., Crise, B., and Burgess, S.M. (2003). Transcription start regions in the human genome are favored targets for MLV integration. *Science* 300, 1749–1751.
 40. Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Bartholomae, C.C., Ranzani, M., Benedicenti, F., Sergi, L.S., Ambrosi, A., Ponzoni, M., et al. (2009). The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J. Clin. Invest.* 119, 964–975.
 41. Hematti, P., Hong, B.K., Ferguson, C., Adler, R., Hanawa, H., Sellers, S., Holt, I.E., Eckfeldt, C.E., Sharma, Y., Schmidt, M., et al. (2004). Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. *PLoS Biol.* 2, e423.
 42. Porada, C.D., Atala, A., and Almeida-Porada, G. (2016). In utero stem cell transplantation and gene therapy: rationale, history, and recent advances towards clinical application. *Mol. Ther. Methods Clin. Dev.* 5, 16020.
 43. Batty, P., and Lillicrap, D. (2019). Advances and challenges for hemophilia gene therapy. *Hum. Mol. Genet.* 28 (R1), R95–R101.
 44. Gangadharan, B., Parker, E.T., Ide, L.M., Spencer, H.T., and Doering, C.B. (2006). High-level expression of porcine factor VIII from genetically modified bone marrow-derived stem cells. *Blood* 107, 3859–3864.
 45. Doering, C.B., Gangadharan, B., Dukart, H.Z., and Spencer, H.T. (2007). Hematopoietic stem cells encoding porcine factor VIII induce pro-coagulant activity in hemophilia A mice with pre-existing factor VIII immunity. *Mol. Ther.* 15, 1093–1099.
 46. Shi, Q. (2018). Platelet-targeted gene therapy for hemophilia. *Mol. Ther. Methods Clin. Dev.* 9, 100–108.
 47. Miao, C.H. (2016). Hemophilia A gene therapy via intraosseous delivery of factor VIII-lentiviral vectors. *Thromb. J.* 14 (Suppl 1), 41.
 48. Perrin, G.Q., Herzog, R.W., and Markusic, D.M. (2019). Update on clinical gene therapy for hemophilia. *Blood* 133, 407–414.
 49. Peyvandi, F., and Garagiola, I. (2019). Clinical advances in gene therapy updates on clinical trials of gene therapy in haemophilia. *Haemophilia* 25, 738–746.
 50. Chandler, R.J., Sands, M.S., and Venditti, C.P. (2017). Recombinant adeno-associated viral integration and genotoxicity: insights from animal models. *Hum. Gene Ther.* 28, 314–322.
 51. Chan, J.K.Y., Gil-Farina, I., Johana, N., Rosales, C., Tan, Y.W., Ceiler, J., McIntosh, J., Ogden, B., Waddington, S.N., Schmidt, M., et al. (2019). Therapeutic expression of human clotting factors IX and X following adeno-associated viral vector-mediated intrauterine gene transfer in early-gestation fetal macaques. *FASEB J.* 33, 3954–3967.
 52. Almeida-Porada, G., Waddington, S.N., Chan, J.K.Y., Peranteau, W.H., MacKenzie, T., and Porada, C.D. (2019). In utero gene therapy consensus statement from the IFeTIS. *Mol. Ther.* 27, 705–707.
 53. Delo, D.M., De Coppi, P., Bartsch, G., Jr., and Atala, A. (2006). Amniotic fluid and placental stem cells. *Methods Enzymol.* 419, 426–438.
 54. Dooriss, K.L., Denning, G., Gangadharan, B., Javazon, E.H., Spencer, H.T., and Doering, C. (2009). Comparison of factor VIII transgenes bioengineered for improved expression in gene therapy of hemophilia A. *Hum. Gene Ther.* 20, 465–478.
 55. Hoeben, R.C., Fallaux, F.J., Cramer, S.J., van den Wollenberg, D.J., van Ormondt, H., Briët, E., and van der Eb, A.J. (1995). Expression of the blood-clotting factor-VIII cDNA is repressed by a transcriptional silencer located in its coding region. *Blood* 85, 2447–2454.
 56. Fallaux, F.J., Hoeben, R.C., Cramer, S.J., van den Wollenberg, D.J., Briët, E., van Ormondt, H., and van Der Eb, A.J. (1996). The human clotting factor VIII cDNA contains an autonomously replicating sequence consensus- and matrix attachment region-like sequence that binds a nuclear factor, represses heterologous gene expression, and mediates the transcriptional effects of sodium butyrate. *Mol. Cell. Biol.* 16, 4264–4272.

57. Majewska, M., Lipka, A., Paukszto, L., Jastrzebski, J.P., Myszczyński, K., Gowkielewicz, M., Jozwik, M., and Majewski, M.K. (2017). Transcriptome profile of the human placenta. *Funct. Integr. Genomics* 17, 551–563.
58. Östling, H., Kruse, R., Helenius, G., and Lodefalk, M. (2019). Placental expression of microRNAs in infants born small for gestational age. *Placenta* 81, 46–53.
59. Majewska, M., Lipka, A., Paukszto, L., Jastrzebski, J.P., Szeszko, K., Gowkielewicz, M., Lepiarczyk, E., Jozwik, M., and Majewski, M.K. (2019). Placenta transcriptome profiling in intrauterine growth restriction (IUGR). *Int. J. Mol. Sci.* 20, E1519.
60. Baglio, S.R., Devescovi, V., Granchi, D., and Baldini, N. (2013). MicroRNA expression profiling of human bone marrow mesenchymal stem cells during osteogenic differentiation reveals Osterix regulation by miR-31. *Gene* 527, 321–331.
61. Martin, E.C., Qureshi, A.T., Dasa, V., Freitas, M.A., Gimble, J.M., and Davis, T.A. (2016). MicroRNA regulation of stem cell differentiation and diseases of the bone and adipose tissue: perspectives on miRNA biogenesis and cellular transcriptome. *Biochimie* 124, 98–111.
62. Khoury, O., Atala, A., and Murphy, S.V. (2020). Stromal cells from perinatal and adult sources modulate the inflammatory immune response in vitro by decreasing Th1 cell proliferation and cytokine secretion. *Stem Cells Transl. Med.* 9, 61–73.
63. Maier, C.L., and Pober, J.S. (2011). Human placental pericytes poorly stimulate and actively regulate allogeneic CD4 T cell responses. *Arterioscler. Thromb. Vasc. Biol.* 31, 183–189.
64. Prevosto, C., Zancolli, M., Canevali, P., Zocchi, M.R., and Poggi, A. (2007). Generation of CD4⁺ or CD8⁺ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. *Haematologica* 92, 881–888.
65. Soland, M.A., Bego, M.G., Colletti, E., Porada, C.D., Zanjani, E.D., St Jeor, S., and Almeida-Porada, G. (2012). Modulation of human mesenchymal stem cell immunogenicity through forced expression of human cytomegalovirus us proteins. *PLoS ONE* 7, e36163.
66. Morandi, F., Raffaghello, L., Bianchi, G., Meloni, F., Salis, A., Millo, E., Ferrone, S., Barnaba, V., and Pistoia, V. (2008). Immunogenicity of human mesenchymal stem cells in HLA-class I-restricted T-cell responses against viral or tumor-associated antigens. *Stem Cells* 26, 1275–1287.
67. Iwaszko, M., and Bogunia-Kubik, K. (2011). Clinical significance of the HLA-E and CD94/NKG2 interaction. *Arch. Immunol. Ther. Exp. (Warsz.)* 59, 353–367.
68. Sullivan, L.C., Clements, C.S., Rossjohn, J., and Brooks, A.G. (2008). The major histocompatibility complex class Ib molecule HLA-E at the interface between innate and adaptive immunity. *Tissue Antigens* 72, 415–424.
69. Braud, V.M., and McMichael, A.J. (1999). Regulation of NK cell functions through interaction of the CD94/NKG2 receptors with the nonclassical class I molecule HLA-E. *Curr. Top. Microbiol. Immunol.* 244, 85–95.
70. Borrego, F., Ulbrecht, M., Weiss, E.H., Coligan, J.E., and Brooks, A.G. (1998). Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187, 813–818.
71. Kaufman, R.J. (1998). Post-translational modifications required for coagulation factor secretion and function. *Thromb. Haemost.* 79, 1068–1079.
72. Mokhtari, S., Colletti, E., Yin, W., Sanada, C., Lamar, Z., Simmons, P.J., Walker, S., Bishop, C., Atala, A., Zanjani, E.D., et al. (2018). A human bone marrow mesodermal-derived cell population with hemogenic potential. *Leukemia* 32, 1575–1586.
73. Sanada, C., Kuo, C.J., Colletti, E.J., Soland, M., Mokhtari, S., Knovich, M.A., Owen, J., Zanjani, E.D., Porada, C.D., and Almeida-Porada, G. (2013). Mesenchymal stem cells contribute to endogenous FVIII:c production. *J. Cell. Physiol.* 228, 1010–1016.
74. Brown, H.C., Wright, J.F., Zhou, S., Lytle, A.M., Shields, J.E., Spencer, H.T., and Doering, C.B. (2014). Bioengineered coagulation factor VIII enables long-term correction of murine hemophilia A following liver-directed adeno-associated viral vector delivery. *Mol. Ther. Methods Clin. Dev.* 1, 14036.