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Transamniotic Delivery of Coagulation Factor VIII mRNA: A Step Toward a Potential Novel Strategy for the Perinatal Management of Hemophilia A

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ABSTRACT

Hemophilia A is an X-linked monogenic disease resulting in insufficient pro-coagulant factor VIII (FVIII) levels. Hemophiliac infants are at risk for life-threatening hemorrhage, especially during birth. No perinatal treatment for Hemophilia A is currently available. It has been previously shown that the transamniotic route is a viable option to deliver exogenous mRNA to the fetus. We sought to determine whether FVIII mRNA so delivered could be translated by the fetus, leading to the presence of FVIII in the fetal circulation. Time-dated pregnant Sprague Dawley dams underwent volume-matched intra-amniotic injections in all their fetuses ($n = 166$) of either a human FVIII (hFVIII) mRNA encapsulated by lipopolyplex (mRNA; $n = 115$) or of the same lipopolyplex without mRNA (control; $n = 51$) on gestational day 17 (E17; term = E21–22). Fetal liver and serum samples were procured daily until term and screened for hFVIII protein by ELISA. There was no maternal mortality. Overall survival was 90% (149/166). Controlled by the mRNA-free injections, fetal serum levels of hFVIII were statistically significantly higher overall in the mRNA group ($p = 0.002$), peaking at E20 (24.4 ± 2.4 ng/mL in the mRNA group vs. 10.5 ± 1.9 ng/mL for control; $p < 0.001$). In the fetal liver, there was variability in statistically significant differences between the groups, with the shorter time point showing significance ($p = 0.003$). Encapsulated exogenous mRNA encoding for factor VIII can be incorporated and translated by the fetus following simple intra-amniotic injection in a rat model. Transamniotic mRNA delivery could become a novel strategy for the perinatal management of Hemophilia A.

1 | Introduction

Hemophilia A is one of the most common inherited bleeding disorders with an incidence of 1:5000 males. This monogenic X-linked disease results from deficiency of pro-coagulant factor VIII (FVIII) levels or function [1]. It can be diagnosed prenatally via amniocentesis, chorionic villus sampling, or cell-free fetal DNA in peripheral maternal blood [2]. Infants with Hemophilia A are at increased risk of intracranial hemorrhage and other potentially severe and life-changing bleeding complications, especially during birth as it can lead to sudden changes in brain perfusion and intracranial pressure [3]. Besides established but limited FVIII replacement therapies, messenger RNA (mRNA) therapies have been recently developed experimentally as novel treatment strategies for this disease [4]. These options, however, are only viable postnatally. No prenatal or intra-partum treatment for Hemophilia A is currently available.

More recently, transamniotic nucleic acid therapy (TRANAT) has emerged experimentally as a minimally invasive method of

fetal mRNA delivery for protein replacement therapies [5]. It has been shown that exogenous mRNA delivered into the amniotic fluid can reach the fetal circulation and therefore various fetal anatomical sites, including the liver, via hematogenous routing from the placenta, besides the widely known intake from plain fetal swallowing and aspiration [5]. A simple amniocentesis is evidently much less invasive than fetal intravascular or even intra-placental injections. In this study, we sought to determine whether FVIII mRNA so delivered could be translated by the fetus, leading to the presence of FVIII in the fetal circulation.

2 | Materials and Methods

This study was approved by the Boston Children's Hospital Institutional Animal Care and Use Committee under protocol 00-00-1369.

2.1 | mRNA Formulation and Encapsulation

A human FVIII (hFVIII) mRNA was commercially obtained following a custom order and sequence optimization process (GenBank: K01740.1; Ribo Pro, The Netherlands). The final sequence consisted of 7056 nucleotides and included proprietary modifications to enhance mRNA translation efficiency (Ribo Pro). Specifically, a methyl group was added to the first nucleotide on the 5′-end, creating a Cap 1 structure to further increase translation efficiency. Also, the 3′-end was elongated by the addition of 150 adenosine monophosphates, as a longer poly (A) tail has been shown to achieve higher protein expression levels than the conventional poly (A) tail of 64 nucleotides [6]. The final mRNA product was then kept at −80°C in RNase-free water at 1 μg/μL.

To minimize degradation following delivery in vivo, the mRNA was encapsulated into a semi-synthetic envelope consisting of a lipid- and cationic polymer-based composite (a so-called lipopolyplex) just prior to injection. Encapsulation was performed using a commercially available TransIT mRNA transfection kit (Mirus Bio, Madison, WI) consisting of two components, a TransIT Reagent proper and a mRNA Boost Reagent, as per the manufacturer's instructions. Briefly, 1 μg of mRNA (1 μg/μL stock) was first suspended in 45 μL of phosphate-buffered saline (PBS). Then, 2 μL of mRNA Boost Reagent and 2 μL of TransIT Reagent were added to the mixture, carefully pipetted, and incubated for 5–10 min at room temperature so as to allow for self-assembling mRNA encapsulation into the lipopolyplex prior to injection in vivo.

2.2 | mRNA Lipopolyplex Encapsulation Efficiency

The encapsulation efficiency of the mRNA into the lipopolyplex was quantified using an ultrasensitive fluorescent nucleic acid stain-based Quant-iT RiboGreen assay (Thermo Fisher Scientific, Waltham, MA) as previously described [7]. Briefly, samples of naked hFVIII mRNA in PBS (1 μg/50 μL stock), of hFVIII mRNA encapsulated using TransIT into lipopolyplexes (1 μg/50 μL stock), and of mRNA standards were diluted with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5; Thermo Fisher Scientific, Waltham, MA) and placed in a 96-well plate. Then, fluorescent RiboGreen reagent (Thermo Fisher) was also diluted with TE buffer and added to the wells. Following a 5-min incubation at room temperature and protected from light, fluorescence intensity was measured on a microplate reader (BMG Labtech, Cary, NC) at 490 nm and 520 nm excitation and emission, respectively, in order to quantify the amount of free mRNA in solution. Encapsulation efficiency was calculated by subtracting the amount of free mRNA from the known total originally used to generate the mRNA lipopolyplexes and expressed as a percentage.

2.3 | Intra-Amniotic Injections

An overview of the experimental design is shown in Figure 1. Thirteen time-dated Sprague Dawley dams (Charles River Laboratories, Wilmington, MA) underwent open intra-amniotic injections under direct vision on gestational day 17 (E17, term = E21–22) as previously described [5]. Briefly, following induction of general anesthesia using 2%–4% inhaled isoflurane (Patterson Veterinary, Greeley, CO) in 100% oxygen, animals were maintained on 2% inhaled isoflurane throughout the entire procedure. In sterile fashion, a midline laparotomy was performed and the pregnant bicornuate uterus was gently eviscerated. All viable fetuses ($n = 166$) received volume-matched (50 μL) intra-amniotic injections of either 1 μg hFVIII mRNA encapsulated into lipopolyplex (mRNA group; $n = 115$) or the same lipopolyplex without any mRNA (control group; $n = 51$) to control for possible inter-species homology of the FVIII protein. Injections were performed using a 33G non-coring needle fitted on a 100 μL syringe (both from the Hamilton Company, Reno, NV) with care to avoid injury to the fetus, placenta, or umbilical cord. The incision was closed in two layers, and powdered metronidazole (Unichem Pharmaceuticals, Hasbrouck Heights, NJ) was applied to the wound. Extended-release buprenorphine Ethiqa XR (Fidelis Animal Health, North Brunswick, NJ) was administered subcutaneously for post-operative analgesia.

2.4 | Specimen Procurement

Dams from both groups were euthanized by CO_2 chamber at daily time points after injection, from E18 to E21 (term). The laparotomy was reopened, and the uterus eviscerated. Each fetus was then removed *en caul*, followed by the opening of the gestational membranes and gross inspection. Normal fetuses (i.e., non-hydropic) underwent sample procurement. First, the umbilical cord was milked toward the fetus to maximize blood return. Fetal blood was then obtained from the subclavian artery and vein under direct vision, followed by the procurement of fetal livers. Maternal blood was obtained via direct intracardiac puncture. All blood samples were centrifuged at 5000 rcf for 8 min to allow for separation of the serum. All samples were then rapidly frozen in dry ice–ethanol baths and stored at −80°C until further processing.

2.5 | hFVIII ELISA

Fetal liver samples were standardized by weight and homogenized in PBS (pH 7.4) containing 1% Triton X-100 (MilliporeSigma, Burlington, MA) with stainless-steel beads

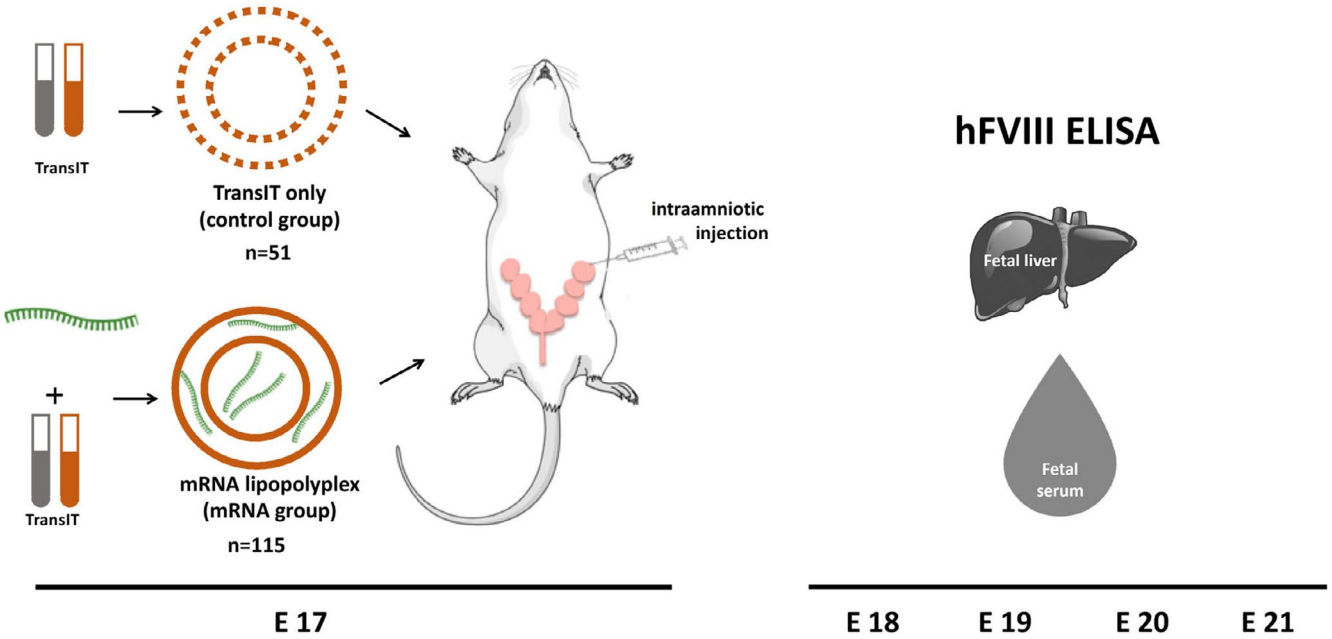

FIGURE 1 | Overview of the experimental protocol. hFVIII = human Factor VIII; TransIT = mRNA encapsulating agent; E = gestational day (term = E21–22).

(Next Advance, Averill Park, NY) in an automated tissue homogenizer (Next Advance) at high speed for 5 min. Liver homogenates were centrifuged at 10,000 rcf for 5 min at room temperature, and the supernatant was then used at a 1:20 dilution for hFVIII protein detection using a commercially available hFVIII ELISA kit (AssayPro, St. Charles, MO) according to the manufacturer's instructions. Fetal and maternal serum samples were standardized by volume and used at a 1:20 dilution for the same ELISA. Final hFVIII concentrations were calculated using a standard curve and expressed in ng/mL. All samples and standards were run in duplicates.

2.6 | Statistical Analyses

Fetal survival comparisons between the groups were calculated by the Fisher's exact test. ELISA data were compared by the nonparametric Wilcoxon rank sum test. Statistical significances were defined as $p < 0.05$. All data were presented as mean ± SEM or as absolute numbers (for fetal survival).

3 | Results

Encapsulation efficiency of the hFVIII mRNA into the lipopolyplex was 97.1%.

3.1 | Survival

Overall fetal survival at all time points was 90% (149/166). Overall survival in the mRNA group at all time points was 87% (100/115), not significantly different when compared to the overall survival of 96% (49/51) in the control group ($p = 0.097$). When comparing fetal survival between the groups at each time point, the only statistically significant difference was observed at term (E21): 62% (16/26) for mRNA vs. 100% (14/14) for control ($p = 0.007$). No significant differences were present at the other time points. At E18: mRNA 97% (33/34) vs. control 100% (15/15; $p = 0.999$). At E19: mRNA 100% (31/31) vs. control 100% (10/10; $p = 0.999$). At E20: mRNA 83% (20/24) vs. control 83% (10/12; $p = 0.999$). There was no maternal mortality and no premature labor in either group.

3.2 | Fetal hFVIII Production

ELISA for the hFVIII protein was positive in samples from the control group, suggesting some degree of human-rat homology for FVIII. However, when controlled by mRNA-free injections, fetal serum levels of hFVIII were statistically significantly higher overall in the mRNA group (16.0 ± 0.9 ng/mL) vs. controls (11.6 ± 1.0 ng/mL; $p = 0.002$). Such a difference peaked at E20 (24.4 ± 2.4 ng/mL in the mRNA group vs. 10.5 ± 1.9 ng/mL for control; $p < 0.001$) (Figure 2).

On the other hand, overall fetal liver hFVIII levels were not statistically different in the mRNA group (38.3 ± 1.1 ng/mL) when compared to control (36.8 ± 1.3 ng/mL; $p = 0.899$). At the same time, there was variability in statistically significant differences between the groups at that site at the different time points, with controls actually showing higher levels at E20 (44.5 ± 2.5 ng/mL vs. 36.1 ± 0.9 ng/mL for mRNA; $p = 0.01$) and at E21 (40.8 ± 1.4 ng/mL vs. 35.5 ± 1.4 ng/mL for mRNA; $p = 0.024$), while the mRNA group showed higher levels at E18 (37.4 ± 2.3 ng/mL vs. 27.2 ± 1.3 ng/mL for control; $p = 0.003$) (Figure 3).

Both groups showed the presence of hFVIII in maternal serum samples; however, no statistical comparisons between the groups could be performed due to the small number of maternal samples for such analysis.

4 | Discussion

Pro-coagulant factor VIII plays an essential role in the intrinsic pathway of the coagulation cascade. It is a large protein consisting of six domains, A1-A2-B-A3-C1-C5, in which the large B domain (40% of the protein) circulates in the blood as a tightly bound complex with von Willebrand factor (vWF) [8, 9]. When activated, FVIII detaches from vWF, and its heavy chain (A1-A2 domains) and light chain (A3-C1-C2 domains) bind together in a calcium-dependent manner to create an activated FVIII complex. This complex then acts as a cofactor of activated factor IX in the activation of factor X and subsequently stimulates the coagulation cascade response [10, 11]. A secondary role of FVIII in hemostasis is the regulation of vWF, which facilitates the adhesion of platelets to the injured vessel wall [12, 13].

A mutation in a gene coding for FVIII causes Hemophilia A. Up to 70% of patients have a severe, life-threatening variant of this disease due to having less than 1% of normal plasma levels of FVIII activity [14, 15]. Current standard of care is based upon administration of FVIII concentrates either as prophylaxis or on demand [16]. Treatment is life-long and often requires frequent injections due to the short half-life of only 8–12 h. Production of FVIII is very costly and available products may lead to undue immune responses [17, 18].

The current lack of prenatal treatment options for Hemophilia A places fetuses and infants at particularly high risk for complications, especially during birth [3]. Although gene therapies to introduce a functional FVIII gene have been explored experimentally both pre- and postnatally, this strategy carries the risk of possible introduction of oncogenic mutations and other genotoxicities, along with immune responses to viral vectors and/or transgenes [18, 19]. mRNA-based strategies for Hemophilia A have recently emerged as potential new alternatives, so far only in the postnatal period [4, 19, 20]. Besides the lower costs and overall higher safety profile of mRNA therapies when compared with direct gene manipulations, the

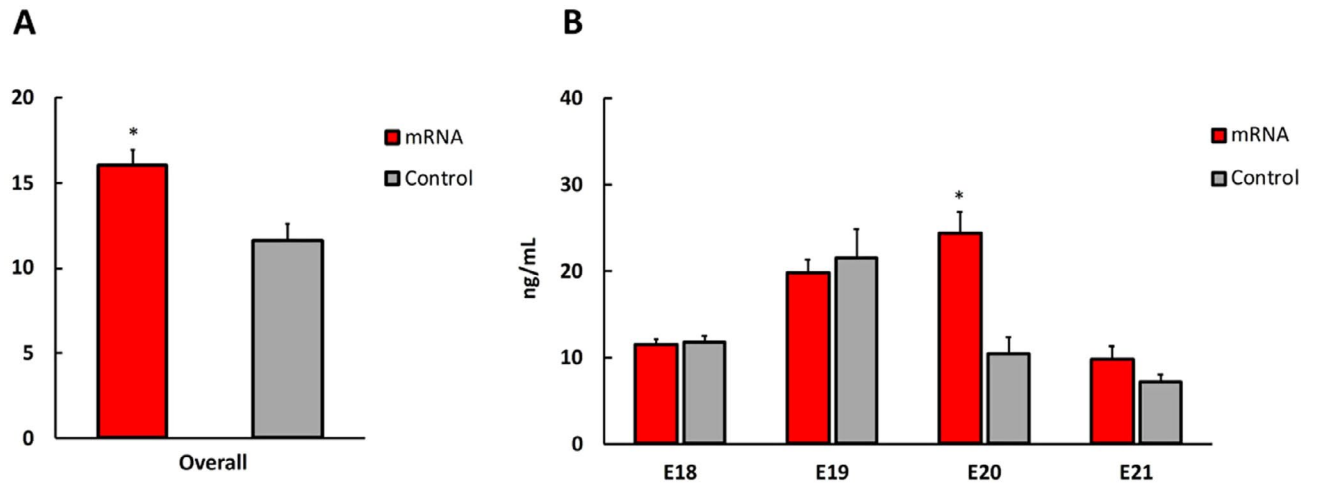

FIGURE 2 | Human Factor VIII (hFVIII) levels in fetal rat sera from the mRNA and Control groups: (A) overall and (B) from E18 to E21. The control group shows presence of hFVIII due to known intespecies homology for that protein. Data presented as mean ± SEM, $*p < 0.05$ for mRNA vs. Control. E = gestational day (term = E21–22).

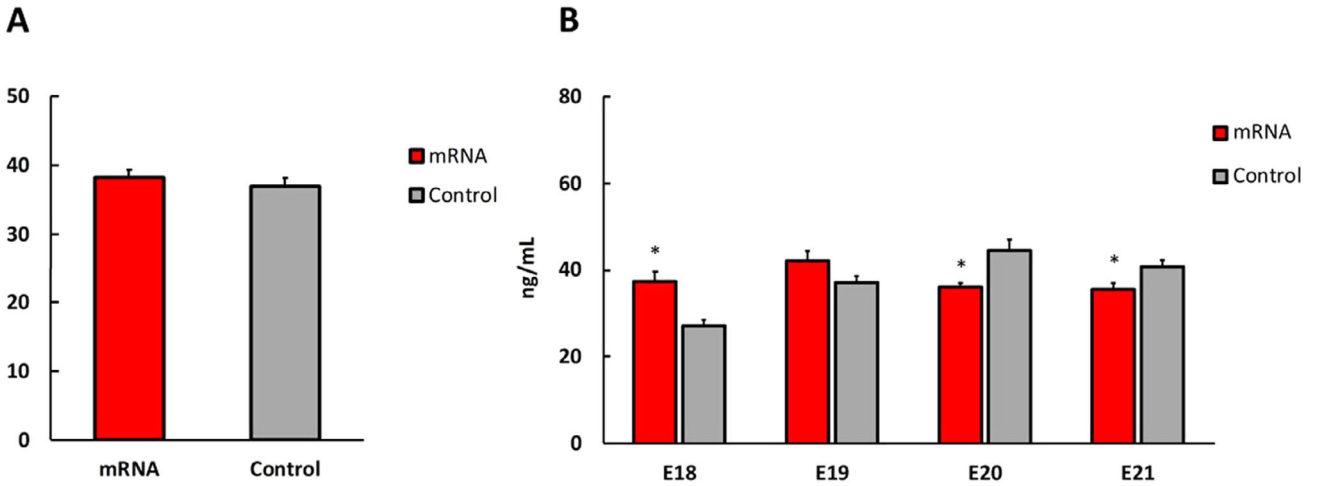

FIGURE 3 | Human Factor VIII (hFVIII) levels in fetal rat livers from the mRNA and Control groups: (A) overall and (B) from E18 to E21. The control group shows presence of hFVIII due to known intespecies homology for that protein. Data presented as mean ± SEM, $*p < 0.05$ for mRNA vs. Control. E = gestational day (term = E21–22).

delivered mRNA undergoes native/intrinsic post-translation processing that tends to decrease the risk of eventual immune response against the final protein product, including FVIII [21].

Here, we have shown that a simple intra-amniotic injection may be a minimally invasive alternative for the delivery of FVIII to the fetus. At the same time, this study was not without notable limitations, arguably to be expected in what is, to our knowledge, a first of its kind. Although the overall fetal serum levels of hFVIII were statistically significantly higher in the mRNA group than in controls, the time-based analysis showed that only at one time point—E20—were the fetal serum levels of hFVIII significantly increased against controls. This was approximately 72 h after the injection. This timeline is consistent with the typically short-lived production of proteins encoded by exogenous mRNA, yet it could still be relevant clinically, especially when labor is anticipated and a simple elective amniocentesis can be planned accordingly. One could envision a number of ways to enhance this principle in future studies, for example by implementing known mRNA codon optimization techniques that can extend the encoded protein half-life by fusion of FVIII to the Fc fragment of human IgG1 [22]. Of note, the levels of hFVIII that we observed in fetal serum were encouraging given that even FVIII restorations to as little as 3%–5% of normal levels can already be very protective [23].

To a good extent, the above-mentioned limitation of this study may also have been related to the fact that control rat fetus showed some presence of hFVIII on ELISA. This is not exactly surprising given the known interspecies homology of FVIII, with up to 51% congruence in amino acid sequences between human and rat FVIII [24]. To our knowledge, available information on commercially available ELISA kits does not specify whether said congruence impacts the accuracy of the kit or not. This is, in fact, why a control group receiving no mRNA had to be included. While an (optional) additional control group receiving scrambled mRNA could have been considered, controlling for interspecies homology had therefore to take precedent. Said homology seemed particularly relevant to our liver analyses. Only at the first time point, that is, 24 h following the intra-amniotic injection, were hFVIII levels in fetal liver significantly higher in the mRNA group. The subsequent time points show wide variability, with the control group showing even higher levels at a few of them. Besides the possibility of a shorter-lived exogenous mRNA translation at that site, hemolysis is known to interfere with ELISA and can cause false positives due to the peroxidase activity of hemoglobin [25, 26]. Our fetal liver samples were washed in PBS to remove gross blood contamination; however, the liver is a highly perfused organ, which, compounded by the maturing fetal hematopoiesis and rising hemoglobin levels of the later days of gestation, could have more significantly affected the ELISA results in that organ.

Of note, under physiologic conditions, both liver and endothelial cells produce the majority of FVIII, while FVIII has been detected in many other cells and tissues as well [27]. Unlike proteins that are defective in other organ-specific genetic diseases, in Hemophilia A, newly synthesized FVIII is not required to occur mainly in the liver [23]. Furthermore, others have shown that when FVIII mRNA was encapsulated using TransIT, as we have done, and injected intravenously in the mouse model of Hemophilia A, occasional signals in the marginal zone of the spleen were observed without any detection of FVIII production in the liver, suggesting that hepatocytes are not the main target for TransIT [28]. More detailed topographic mapping of mRNA incorporation/translation was beyond the scope of this experiment; however, further research in this direction is warranted. Dosage optimization, including amount, timing in relation to birth, and eventual repeat injections, preferably in a large animal model, would also be necessary before clinical translation could be contemplated, as would comparisons between different encapsulating agents. Finally, our use of a healthy model, justifiable in a first study on a completely novel approach such as this, prevented functional clotting comparisons.

These limitations notwithstanding, and to be expected on an introductory study, our data show that exogenous encapsulated mRNA encoding for human pro-coagulation factor VIII appears to be incorporated and translated by the feto-placental unit following simple intra-amniotic injection in a healthy rat model. Topographic mapping of mRNA incorporation/translation remains to be determined in a model with minimal/no human homology. Transamniotic nucleic acid therapy could become a novel strategy for the perinatal management of Hemophilia A.

Author Contributions

Conceptualization: K.M., D.O.F. Data Curation: K.M., E.M.S., T.T.D., B.S.B., Y.V.S., E.Z. Formal Analysis: K.M., D.Z., D.O.F. Funding acquisition: D.O.F. Investigation: K.M., E.M.S., T.T.D., D.O.F. Methodology: K.M., D.Z., D.O.F. Project administration: K.M., D.O.F. Resources: K.M., D.O.F. Software: K.M., D.Z. Supervision: D.O.F. Validation: K.M., D.Z., D.O.F. Visualization: K.M., D.O.F. Writing-original draft: K.M., D.Z. Writing-review and editing: K.M., D.O.F.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All raw data are available upon request.

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