Journal of Controlled Release 374 (2024) 415–424

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Transplacental delivery of factor IX Fc-fusion protein ameliorates bleeding phenotype of newborn hemophilia B mice

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ARTICLE INFO

Keywords:
Factor IX
Adenovirus vector
Transplacental delivery
Hemophilia

ABSTRACT

Hemophilia B is an inherited hemorrhagic disorder characterized by a deficiency of blood coagulation factor IX (FIX) that results in abnormal blood coagulation. The blood coagulation is already evident in hemophiliacs at the fetal stage, and thus intracranial hemorrhage and other bleeding complications can occur at birth, leading to sequelae. Therefore, it is important to develop effective treatments for hemophiliacs *in utero*. In this study, in order to transplacentally deliver FIX from pregnant mice to their fetuses, an improved adenovirus (Ad) vector expressing human FIX fused with the IgG Fc domain (FIX Fc fusion protein), which plays a crucial role in neonatal Fc receptor (FcRn)-mediated transcytosis across the placenta, was intravenously administered to E13.5 pregnant mice. Significant levels of FIX Fc fusion protein were detected in 0-day-old newborn mice whose mothers were administered an Ad vector expressing FIX Fc fusion protein. Wild-type FIX overexpressed in the pregnant mice was not delivered to the fetuses. Plasma FIX levels in the newborn mice were relatively well correlated with those in their mothers, although transplacental delivery efficiencies of FIX Fc fusion protein were slightly reduced when the FIX Fc fusion protein was highly expressed in the mother mice. Plasma FIX levels in the newborn mice were about 3.6–6.4% of those in their mothers, Transplacental delivery of FIX Fc fusion protein to their fetuses successfully improved the blood clotting ability in the newborn mice.

1. Introduction

Hemophilia B is a serious genetic disorder which is caused by mutations in the coagulation factor IX (FIX) gene in the X chromosome. The FIX-gene mutations lead to abnormally low FIX clotting activity. More than 30,000 people suffer from hemophilia B worldwide [1]. Several types of hemophilia B therapeutics, including recombinant FIX protein and antibody medicines, have been developed so far [2]. An adeno-associated virus (AAV) serotype 5 vector expressing FIX was approved by the U.S. Food and Drug Administration (FDA) in 2022, followed by approval in Europe in 2023 [3].

Hemophilia patients already exhibit abnormal blood coagulation *in utero*, and thus there is a risk that intracranial and extracranial hemorrhage and other bleeding complications could occur at birth in response to stimuli such as external pressure at the birth canal, leading to sequelae [4–6]. The incidence of intracranial and extracranial hemorrhage in hemophilia neonates has been estimated as 3.58% [7]. Several types of hemophilia B therapeutics have been developed, as described above; however, it remains a major challenge to develop an effective hemophilia treatment for the fetus *in utero*. And while *in utero* gene therapy studies, including studies involving genome editing, have been actively performed for the treatment of various genetic diseases [8–10], many problems with such therapies remain, including issues related to safety and ethics. Further studies are necessary before *in utero* gene

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https://doi.org/10.1016/j.jconrel.2024.08.022
Received 22 December 2023; Received in revised form 21 July 2024; Accepted 13 August 2024
Available online 24 August 2024

therapy can be practically applied in humans.

The placenta is a crucial organ that exchanges substances between maternal and fetal blood, but the blood-placental barrier, which is composed of multiple layers of cells, restricts the transfer of all but a few proteins, with immunoglobulin G (IgG) being one of the exception of several proteins. Neonatal Fc receptor (FcRn), which is mainly expressed in syncytiotrophoblast, transfers IgG from maternal blood to fetal blood by transcytosis, thereby protecting neonates from various types of pathogens [11]. IgG is efficiently transferred from mother to fetus. Although fetal IgG levels remain low until the second trimester, they gradually increase as gestation proceeds [12]. Fetal IgG levels become similar or higher than maternal IgG levels just before delivery. These data led us to hypothesize that fusion of the Fc region of IgG to FIX makes it possible for FIX to be transplacentally delivered to the fetus.

In this study, we developed an improved adenovirus (Ad) vector expressing the FIX gene fused with the Fc region for transplacental delivery of FIX from pregnant mice to their fetuses. Intravenous administration of the Fc-fused FIX-expressing Ad vector in E13.5 pregnant mice achieved therapeutic levels (more than 1% of the normal level) of plasma FIX in the newborn mice and significantly improved the blood clotting activities of neonatal hemophilia B mice. On the other hand, when wild-type FIX was overexpressed in the pregnant mice, detectable levels of plasma FIX were not found in the neonatal mice.

2. Materials and methods

2.1. Cells

HEK293 cells (a human embryonic kidney cell line) and Huh-7 cells (a human well-differentiated hepatocellular carcinoma cell line, JCRB0403, obtained from the JCRB Cell Bank, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2–95% air.

2.2. Plasmids and Ad vectors

The human FIX gene (kindly provided by Tomohiko Yamasaki, National Institute for Materials Science, Tsukuba, Japan) fused with the Fc region of IgG was produced using an In-Fusion® HD Cloning Kit (Takara Bio, Otsu, Japan). Briefly, a gene encoding the Fc region-G4S linker-Fc region (Fc-G4S-Fc gene) was synthesized by artificial gene synthesis (Azenta Life Sciences, South Plainfield, NJ), with reference to the previous study developing the recombinant factor VIII fused with dimeric Fc region [13]. The human FIX gene without stop codon was amplified by PCR using pAHA-hFIX [14] as a template. Homologous recombination between the Fc-G4S-Fc gene and the FIX gene was performed using In Fusion HD Enzyme Premix, producing pAHA-FIXFc. pHMCMV-FIXFc, which contains the FIX gene fused with the Fc-G4S-Fc gene under the cytomegalovirus (CMV) promoter, was constructed by using pHMCMV6 [15] and pAHA-FIXFc. pHMCMV-hFIX was created by using pHMCMV6 and pAHA-hFIX. An Ad vector plasmid, pAdHM4-E4-122aT [16], and pAHAFIX-Fc were digested with I-*Ceu*I and PI-*Sce*I. The resulting fragments were ligated, creating pAd-E4-122aT-AHAFIXFc. Ad vector plasmids, pAdHM15-RGD-CMVFIXFc and pAdHM15-RGD-CMVFIX, were similarly produced by ligation of pAdHM15-RGD [17] and pHMCMV-FIXFc, or pHMCMV-hFIX.

Ad vectors expressing wild-type human FIX or human FIX fused with Fc-G4S-Fc, AdRGD-CMVFIX, Ad-E4-122aT-AHAFIXFc and AdRGD-CMVFIXFc, were produced by an improved *in vitro* ligation method [15,18]. Briefly, an Ad vector plasmid was digested with *Pac*I to release the recombinant viral genome, and was transfected into HEK293 cells plated on 60-mm dishes using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). Ad vectors were propagated in HEK293 cells, purified by two rounds of cesium chloride-gradient ultracentrifugation, dialyzed, and stored at -80 °C. An Ad vector containing an AHA promoter-driven firefly luciferase expression cassette, Ad-E4-122aT-AHAL2, was similarly produced as described above using pAdHM4-E4-122aT. An Ad vector containing a CMV promoter-driven firefly luciferase expression cassette, Ad-E4-122aT-CMVL2, which is identical to Ad-E4-122aT-L2 [16], was previously constructed. An Ad vector containing an AHA promoter-driven FIX expression cassette, Ad-E4-122aT-AHAFIX, was previously produced [14]. Further information about Ad vector preparation is available on request. The virus particles (VPs) were quantified using a spectrophotometric method [19]. Biological titers were measured using an Adeno-X-rapid titer kit (Takara Bio). The ratios of the particle-to-infectious titer were between 5.6 and 14.5 for each Ad vector used in this study.

2.3. Mice

C57BL/6 J mice (5–7 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). FIX gene knockout mice (hemophilia B model mice) were obtained from Jackson Laboratory (Bar Harbor, ME). Ad vectors were intravenously administered *via* a tail vein to E13.5 pregnant mice at the indicated doses. All animal experimental procedures used in this study were approved the Animal Experiment Committee of Osaka University (doyakuR03–1-1), and were performed in accordance with the institutional guidelines for animal experiments at Osaka University.

2.4. Evaluation of firefly luciferase expression in cultured cells following Ad vector transduction

Huh-7 cells were seeded on a 96-well plate at a density of 1×10^4 cells/well. On the following day, cells were transduced with Ad-E4-122aT-AHAL2 and Ad-E4-122aT-CMVL2 at the indicated multiplicities of infection (MOIs) for 24 h. Firefly luciferase activities in the cells were measured using a luciferase assay system (PicaGene LT2.0; Toyo Inki, Tokyo, Japan) after a total 48-h incubation.

2.5. Evaluation of FIX expression in mouse plasma

Blood samples were collected on the indicated days by retro-orbital bleeding in mother mice or venous bleeding in neonatal mice. Plasma FIX activities were determined as previously described [14]. In brief, plasma FIX levels were measured using an AssayMax Human Factor IX ELISA Kit (ASSAYPRO, St. Charles, MO) or were sensitively measured according to the method of Barzel et al. [20]. First, 100 μL of mouse anti-human FIX antibody (F2645; Sigma-Aldrich, St. Louis, MO) which was diluted by 1000-fold in carbonate-bicarbonate buffer (Sigma-Aldrich) was added to 96 well ELISA plates (Nunc, Rochester, NY) and was incubated overnight at 4 °C. After washing with PBS-T, the plates were blocked with Immunoblock (DS Pharma Biomedical Co., Osaka, Japan) for 1 h at room temperature. After washing with PBS-T, plasma diluted 100–10,000-fold was added to the wells, and the plates were incubated for 2 h at 37 °C. After washing with PBS-T, 100 μL of horseradish peroxidase (HRP)-labeled goat anti-human FIX antibody (4200-fold diluted; Affinity Biologicals, Ancaster, Canada) was added, followed by incubation at 37 °C for 2 h. TMB ELISA peroxidase substrate (Thermo Fisher Scientific) was added, followed by incubation for 15 min at room temperature. The reaction was then stopped by adding 0.5 N HCl. Absorbance was measured at 450 nm.

2.6. Measurement of Ad vector genome copy numbers and human FIX mRNA levels in the liver

Ad vectors were intravenously administered to wild-type non-pregnant mice at a dose of 5.9×10^{11} IFU/kg. The livers were collected 4 days after administration, followed by isolation of total RNA and DNA from the livers. Determination of Ad vector genome copy numbers and

human FIX mRNA levels in the liver was performed as previously described [21]. The sequences of the primers are described in Supplemental Table 1.

2.7. Analysis of hepatotoxicity after Ad vector administration

Ad vectors were intravenously administered to wild-type non-pregnant mice at a dose of 5.9×10^{11} IFU/kg. Blood samples were collected on the indicated days by retro-orbital bleeding following Ad vector administration. Serum samples were collected by centrifugation. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using a Fuji Dri-Chem Slide kit (Fujifilm, Tokyo, Japan).

2.8. Western blotting analysis

Plasma proteins were denatured by adding 5× Sample buffer (250 mM Tris-HCl, pH 6.8, 30% 2-mercaptoethanol, 10% SDS, 20% glycerol) to 50-fold diluted mouse plasma, followed by incubation at 98 °C for 5 min. The samples were analyzed by SDS-PAGE, then electro-transferred onto a PVDF membrane. After blocking the membrane with 5% skim milk-containing TBS-T for 1 h, the membrane was incubated with goat anti-human FIX (GAFIX; 1:100, Affinity Biologicals) and goat anti-human IgG antibodies (1:200, Jackson ImmunoResearch) at 4 °C overnight. After washing the membrane with TBS-T, the membrane was incubated with HRP-labeled anti-goat IgG antibody (A5420; Sigma-Aldrich). Images were captured using Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) and LAS3000 (Fujifilm).

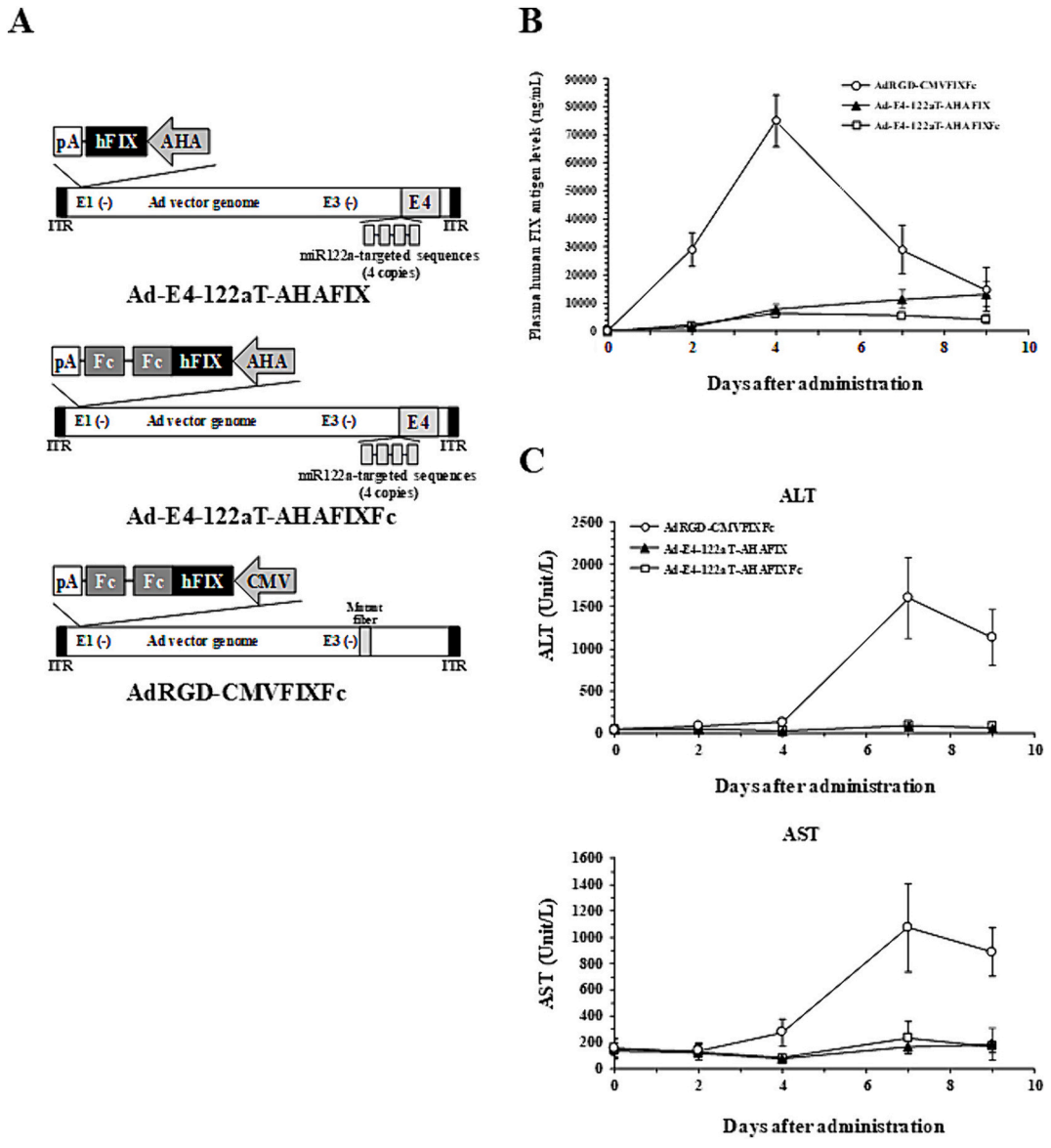

Fig. 1. Transduction properties of Ad vectors in mice following intravenous administration. (A) A schematic diagram of Ad vectors used in this study. Wild-type human FIX or human FIX fused with the Fc domain of the IgG expression cassette was incorporated into the E1-deleted region of the Ad vector genome. ITR, inverted terminal repeat; hFIX, human factor IX; Fc, the Fc region of IgG; pA, bovine growth hormone poly A signal. CMV, cytomegalovirus immediate-early promoter; AHA, synthetic liver-specific promoter composed of the apolipoprotein E enhancer, the hepatocyte control region, and the human α1-antitrypsin promoter. (B, C) Plasma human FIX antigen levels (B) and serum ALT and AST levels (C) in wild-type mice following intravenous administration of Ad vectors. Wild-type mice were intravenously administered Ad vectors at a dose of 5.9×10^{11} IFU/kg. Plasma samples were collected *via* retro-orbital bleeding, followed by determination of human FIX antigen levels. The plasma samples on day 0 were recovered from the mice before Ad vector administration. Results are shown as average ± S.D. (n = 5–6).

2.9. Tail tip survival test

Tail tip survival test was performed as previously described [22]. Briefly, Ad-E4-122aT-AHAFIXFc and Ad-E4-122aT-AHAFIX were intravenously administered to E13.5 pregnant hemophilia B mice at a dose of 5.9×10^{11} IFU/kg as described above. The tails of newborn 0-day-old mice were cut off at 5 mm from the tip. Survival of the mice was assessed 24 h later.

2.10. Immunization with human FIX protein following subcutaneous administration

Ad-E4-122aT-AHAFIXFc was intravenously administered to E13.5 pregnant wild-type mice at a dose of 5.9×10^{11} IFU/kg as described above. At the age of 5 weeks, the newborn mice were subcutaneously immunized with human FIX protein at a dose of 5 μg/mouse as previously described [14]. Plasma samples were collected on day 14 after immunization. Titers of plasma anti-human FIX IgG levels were determined by ELISA as previously described [14].

2.11. Statistical analysis

All results are shown as the mean ± S.D. Statistical significance was analyzed by Student's *t*-test or One-way ANOVA followed by Bonferroni multiple comparison test using Graph Pad Prism 5.04 software (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. An Ad vector expressing FIX Fc fusion protein

In order to transplacentally deliver FIX expressed in the liver of pregnant mice to their fetuses, the dimeric human IgG Fc region was fused with FIX using a G4S linker, with reference to the previous study [13], producing FIX Fc fusion protein (Fig. 1A). The gene encoding FIX Fc fusion protein was inserted downstream of the synthetic liver-specific AHA promoter, which was composed of the apolipoprotein E enhancer, the hepatocyte control region, and the human α1-antitrypsin promoter. The FIX Fc fusion protein expression cassette was incorporated in the E1-deleted region in pAdHM4-E4-122aT, which contains four copies of sequences perfectly complementary to liver-specific miR-122aT in the 3′-untranslated region (UTR) of the E4 gene to suppress the leaky expression of the virus genes in the liver. We previously demonstrated that an Ad vector containing four copies of miR-122aT-targeted sequences in the 3'-UTR of the E4 gene showed significantly lower hepatotoxicity and efficient and long-term transgene expression than a conventional Ad vector [16]. An Ad vector expressing FIX Fc fusion protein, Ad-E4-122aT-AHAFIXFc, was normally propagated in HEK293 cells. A fiber-modified Ad vector containing the arginine-glycine-aspartate (RGD) motif in the HI loop of the fiber knob and the CMV promoter-driven FIX Fc fusion protein expression cassette, AdRGD-CMVFIXFc, was similarly produced.

In order to evaluate the human FIX expression levels following intravenous administration of the Ad vectors in mice, the plasma samples were collected from the wild-type mice following Ad vector administration, followed by measurement of human FIX levels in the plasma. AdRGD-CMVFIXFc mediated efficient plasma human FIX levels in the mice with a peak on day 4 (Fig. 1B). The plasma human FIX levels in AdRGD-CMVFIXFc-treated mice gradually declined after day 4. Serum ALT and AST levels were significantly elevated after day 4 in AdRGD-FIXFc-treated mice (Fig. 1C), indicating that AdRGD-CMVFIXFc mediated the hepatotoxicity. Ad-E4-122aT-AHAFIX and Ad-E4-122aT-AHAFIXFc exhibited approximately 8-fold lower levels of plasma human FIX on day 4 than AdRGD-CMVFIXFc, because the CMV promoter mediated the higher promoter activity than the AHA promoter. The human FIX mRNA levels in the liver of AdRGD-CMVFIXFc-injected mice were approximately 70-fold higher than those of Ad-E4-122aT-AHAFIXFc-injected mice on day 4 (Supplementary Fig. 1 A). Comparable levels of Ad vector copy numbers were found in the liver of AdRGD-CMVFIXFc-injected and Ad-E4-122aT-AHAFIXFc-injected mice (Supplementary Fig. 1B). Ad-E4-122aT-CMVL2, which contained a CMV promoter-driven firefly luciferase expression cassette, mediated more than 10-fold higher luciferase expression in Huh-7 cells, which are a human well-differentiated hepatocellular carcinoma cell line, than Ad-E4-122aT-AHAL2 (Supplementary Fig. 2). However, the plasma human FIX levels in Ad-E4-122aT-AHAFIX-treated and Ad-E4-122aT-AHAFIXFc-treated mice were sustained until 9 days after administration. Statistically significant differences in the plasma human FIX levels were not found on day 9 among the 3 types of Ad vectors. No apparent elevation in serum ALT and AST levels was not found in Ad-E4-122aT-AHAFIX-treated and Ad-E4-122aT-AHAFIXFc-treated mice, indicating that Ad-E4-122aT-AHAFIX and Ad-E4-122aT-AHAFIXFc did not induce any apparent hepatotoxicity. Based on these data, Ad-E4-122aT-AHAFIX and Ad-E4-122aT-AHAFIXFc were primarily used in the following experiments.

3.2. FIX Fc fusion protein was transplacentally delivered to the fetus following Ad vector-mediated transduction in pregnant mice

In order to examine whether wild-type human FIX and FIX Fc fusion protein were transplacentally delivered to the fetus, plasma FIX levels in the mother and neonatal mice were measured following intravenous administration of Ad-E4-122aT-AHAFIX and Ad-E4-122aT-AHAFIXFc in E13.5 pregnant wild-type mice (Fig. 2A). Ad-E4-122aT-AHAFIX, which expressed the wild-type human FIX, produced approximately 4000 ng/mL of human FIX in the plasma of the mother mice on the childbirth day (7–8 days after administration), but detectable levels of human FIX were not found in the plasma of the 0-day old newborn mice (Fig. 2B). A human FIX-expressing Ad vector containing the RGD peptide in the HI loop, AdRGD-CMVFIX, in wild-type pregnant mice did not also mediate detectable levels of human FIX in the plasma of neonatal mice (Supplementary Fig. 3). On the other hand, approximately 1000 ng/mL of human FIX was detected in the plasma of the mother mice receiving Ad-E4-122aT-AHAFIXFc on the childbirth day. The plasma of the 0-day-old newborn mice contained approximately 46 ng/mL of human FIX (0.92% of the normal FIX level), which was 4.6% of the human FIX level in the plasma of the mother mouse.

Next, western blotting was performed to examine whether the human FIX in the mother and newborn wild-type mouse plasma possessed the Fc region. Clear bands of approximately 120 kDa, which corresponds to the molecular weight of FIX Fc fusion protein, were detected by anti-human FIX and anti-human IgG antibodies in both mother and neonatal wild-type mouse plasma samples following administration of Ad-E4-122aT-AHAFIXFc (Fig. 2C). An approximately 55 kDa band of human FIX was found by anti-human FIX antibody, but not by anti-human IgG antibody, in the samples of the mother mice receiving Ad-E4-122aT-AHAFIX. These results indicated that FIX Fc fusion protein overexpressed in the mother mice was transferred to the fetus through the placenta.

In order to evaluate the correlation between the concentrations of FIX Fc fusion protein in the plasma of the mother mice on the childbirth day and the 0-day-old newborn mice, plasma FIX levels in the mother and neonatal mice were measured following intravenous administration of the different doses of Ad-E4-122aT-AHAFIXFc in E13.5 pregnant wild-type mice. As the plasma FIX levels in the mother mice increased, the plasma FIX levels in the newborn mice increased (Fig. 2D); however, the ratios of plasma FIX levels between newborn and mother mice gradually decreased as plasma FIX levels in the mother mice increased (Fig. 2E). When plasma FIX levels in the mother mice were less than approximately 600 ng/ml, the efficiencies of transplacental transfer of the FIX Fc fusion protein were 0.0668 (slope of the graph) (Fig. 2F). When mother mice produced plasma FIX at levels greater than

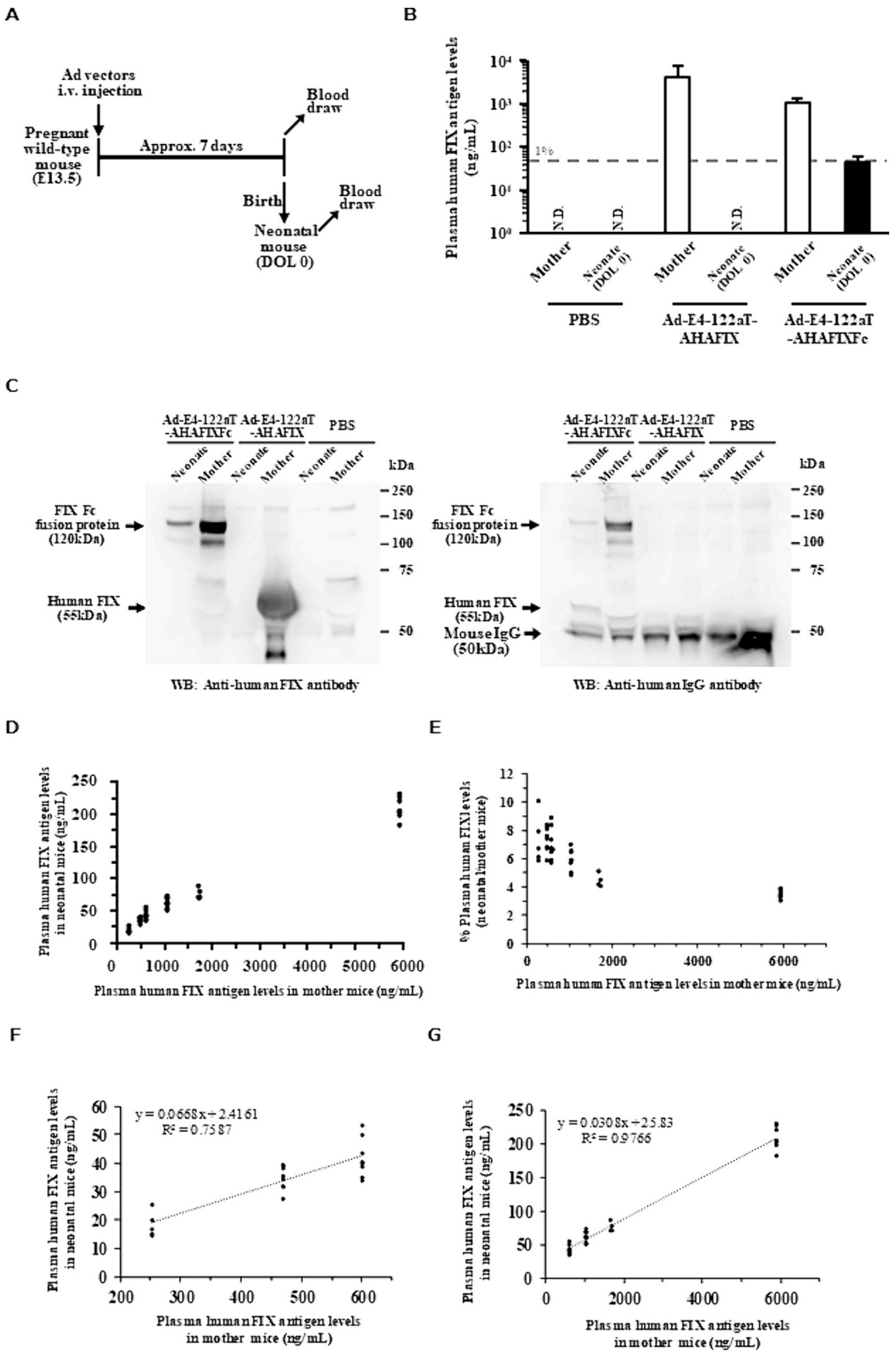

Fig. 2. Plasma human FIX antigen levels in the mother and neonatal mice following intravenous administration of human FIX-expressing Ad vectors. E13.5 pregnant wild-type mice were administered Ad-E4-122aT-AHAFIX and Ad-E4-122aT-AHAFIXFc at a dose of 5.9×10^{11} IFU/kg. Following administration, plasma samples were collected from DOL 0 neonatal mice. (A) The schedule of this experiment. (B) Plasma human FIX antigen levels in the mother and DOL 0 neonatal mice. Results are shown as average ± S.D. ($n = 3$ for mother mice and 8–24 for neonatal mice). DOL: day of life. (C) Western blot analysis of Fc-fused FIX in the plasma of mother and neonatal wild-type mice. Representative images from three independent experiments are shown. (D-G) Correlation between plasma FIX antigen levels in mother and neonatal wild-type mice. (E) The ratios of plasma human FIX antigen levels in the neonatal mice to those in the mother mice. The plasma human FIX antigen levels in the neonatal mice were normalized to those in the mother mice. (F, G) Correlation between plasma FIX antigen levels in the mother mice producing less than 700 ng/ml of human FIX (F) and more than 1000 ng/ml of human FIX (G) and in neonatal mice. N.D.: not detected.

approximately 600 ng/ml, the efficiencies of transplacental transfer of the FIX Fc fusion protein were slightly reduced to 0.0308 (Fig. 2G). These data indicated that although, basically, plasma FIX levels in newborn mice were correlated with those in pregnant mice, the efficiencies of transplacental delivery of FIX Fc fusion protein from pregnant mice to their fetuses gradually decreased when plasma FIX levels in the mother mice were elevated.

3.3. Blood circulation of the transplacentally delivered FIX Fc fusion protein in the newborn mice

Next, in order to evaluate the half-lives of FIX Fc fusion protein in the blood of neonatal mice, plasma FIX protein levels were determined in the newborn mice of each day of age from 2 days of age (Fig. 3A). The plasma FIX Fc fusion protein levels decreased to 49.6% on day 1, and to 23.7% on day 2 (Fig. 3B). These results indicated that the half-life of FIX Fc fusion protein in the blood of neonatal mice was approximately 24 h.

3.4. Phenotype correction of newborn hemophilia B mice by transplacental delivery of FIX Fc fusion protein to the fetus

In order to examine whether transplacental delivery of FIX Fc fusion protein mediated phenotype correction of newborn hemophilia B mice, blood coagulation activity in the newborn hemophilia B mice was evaluated (Fig. 4A). Administration of Ad-E4-122aT-AHAFIXFc to pregnant hemophilia B mice resulted in approximately 86 ng/mL and 1725 ng/mL of plasma FIX levels in the 0-day old newborn and mother mice on the childbirth day, respectively (Fig. 4B). This FIX concentration in the newborn mice corresponded to 1.7% of the normal FIX levels. The blood coagulation activity levels of FIX Fc fusion protein in the plasma of newborn mice were approximately 3.5% of the normal level (Fig. 4C). These results indicated that FIX Fc fusion protein was transplacentally delivered to the fetuses from their mothers and mediated the blood coagulation activity in the blood of newborn hemophilia B mice.

Next, we performed a tail clip survival test of 0-day-old newborn hemophilia B mice. One out of twelve newborn mice of PBS-treated mother mice survived (Fig. 4D). When the pregnant mice were treated with Ad-E4-122aT-AHAFIX, which expressed wild-type human FIX, only 3 of 15 newborn mice survived. On the other hand, 20 of 21 newborn mice from Ad-E4-122aT-AHAFIXFc-treated pregnant mice survived. These data indicated that the hemostatic phenotype of newborn hemophilia B mice was significantly improved by intravenous administration of Ad-E4-122aT-AHAFIXFc in pregnant hemophilia B mice. These data established that Ad-E4-122aT-AHAFIXFc-mediated FIX-Fc delivery to the neonate through pregnant individual successfully provided therapeutic values of FIX coagulation activity during the neonatal period in hemophilia B mice.

3.5. Immune tolerance to human FIX was not induced in the newborn mice by overexpressing FIX Fc fusion protein in the pregnant mice

In order to examine whether immune tolerance to human FIX was induced in the newborn mice by overexpressing FIX Fc fusion protein in the pregnant mice, the wild-type mice were immunized with human FIX protein when the newborn mice became 5 weeks old (Fig. 5A). Comparable levels of anti-human FIX IgG titers were found in the serum of mice from the non-treated mother mice and the mother mice receiving Ad-E4-122aT-AHAFIXFc on day 14 after immunization (Fig. 5B). These data indicated that overexpression of FIX Fc fusion protein in the pregnant mice did not confer immune tolerance to human FIX in the newborn mice.

4. Discussion

In this study, FIX was transplacentally delivered from pregnant mice to their fetuses by overexpressing FIX fused with the IgG Fc domain in the pregnant mice using an improved Ad vector in order to treat the hemostatic phenotype of the hemophilic B fetus. When wild-type FIX was overexpressed in the pregnant wild-type mice, detectable levels of FIX were not found in the newborn mice (Fig. 2B), suggesting that an Ad vector was not delivered transplacentally to the fetus after intravenous administration in pregnant mice. On the other hand, FIX was detected in the neonatal mice when an Ad vector expressing FIX Fc fusion protein was administered to the pregnant wild-type mice. IgG is actively transferred from the mother to the fetus by neonatal Fc receptor (FcRn)-mediated transcytosis. Previous studies reported that several proteins were transplacentally transferred to the fetus by fusing with the IgG Fc domain, while the wild-type forms of the proteins could not be transferred to the fetus [23–25]. Therefore, it could be reasonably considered that FIX Fc fusion protein was transferred from the pregnant mice to the fetus *via* FcRn-mediated transcytosis. As the doses of Ad-E4-122aT-AHAFIXFc increased, plasma levels of human FIX in the mother mice increased, leading to the elevation in the plasma FIX levels in the neonates, because the plasma FIX Fc fusion protein in the mother mice was transferred to the neonates by the neonatal Fc receptor which is expressed in the placenta *via* active transcytosis across the placenta;

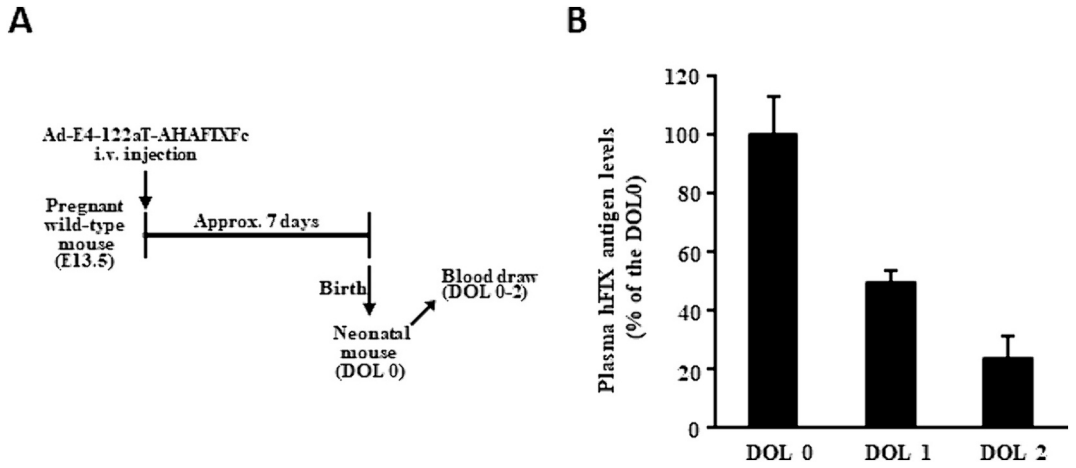

Fig. 3. (A) The schedule of this experiment. (B) Plasma human FIX antigen levels in neonatal mice. E13 pregnant wild-type mice were administered Ad-E4-122aT-AHAFIXFc at a dose of 5.9×10^{11} IFU/kg. Following administration, plasma samples were collected from DOL 0–2 neonatal mice. Mice were sacrificed at each time point. Plasma human FIX antigen levels on each day were normalized by the data of littermates on day 0 as 100%. Results are shown as average ± S.D. (n = 3 for mother mice and n = 5–7 for neonatal mice). DOL: day of life.

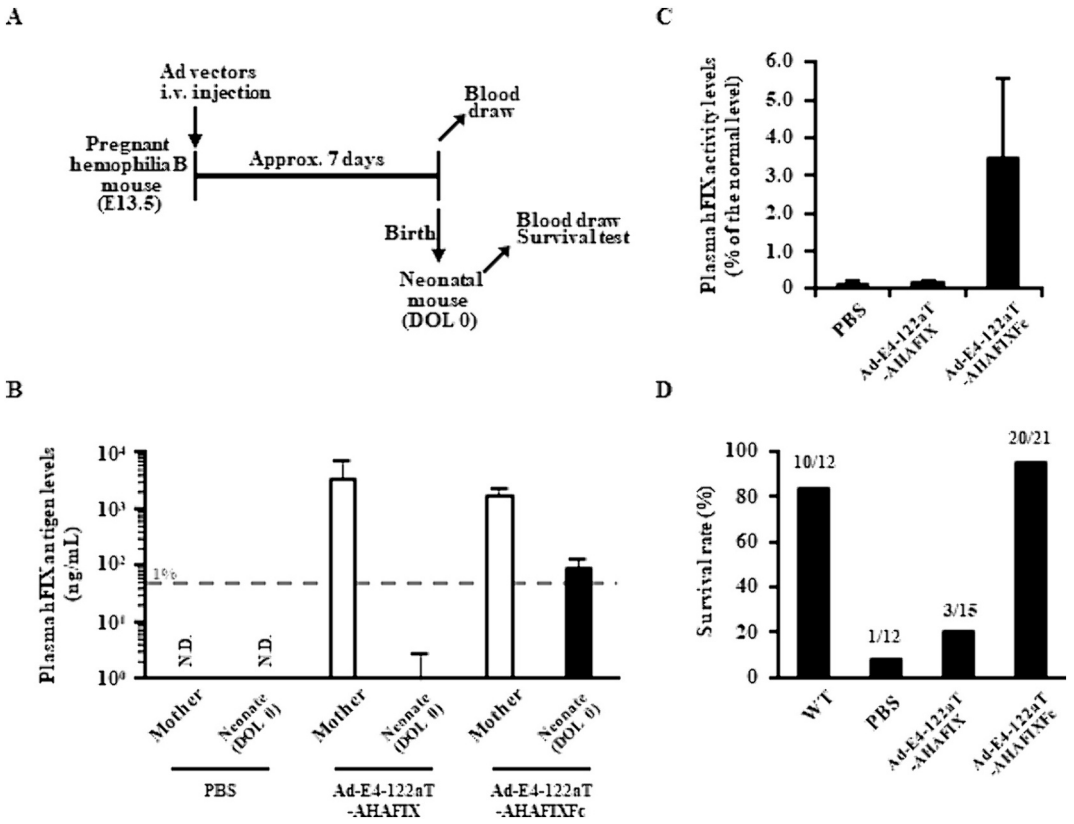

Fig. 4. Therapeutic efficiency of Ad-AHAFIXFc for fetal hemophiliac B mice. (A) The schedule of this experiment. E13 pregnant hemophilia B mice were administered Ad-E4-122aT-AHAFIXFc at a dose of 5.9×10^{11} IFU/kg. Following administration, plasma samples were collected from DOL 0 neonatal mice. (B, C) Plasma human FIX antigen (A) and activity (B) levels in DOL 0 hemophilia B neonatal mice. (D) Survival rates of DOL 0 wild-type and hemophilia B neonatal mice following tail clipping. The survival of neonatal mice was observed 24 h after tail clipping. The numbers of examined and surviving mice are shown at the top of each column. Results are shown as average ± S.D. in panels (B) and (C) (n = 3 for mother mice and 8–21 for neonatal mice). DOL: day of life. N.D.: not detected.

however, as the plasma FIX levels in the mother mice increased, the ratios of FIX-Fc levels in the plasma between the mother mice and neonates decreased, because the neonatal Fc receptor-mediated transcytosis was saturated (Fig. 2D-G).

It is necessary to maintain the plasma FIX concentration at more than 1% (50 ng/ml) of the normal level in order to achieve the therapeutic effects. In this study, 1.7% and 3.5% of the normal plasma FIX concentrations and FIX activity levels, respectively, were found in the 0-day-old hemophilia B newborn mice (Fig. 4B, C), indicating that sufficient levels of plasma FIX in the hemophilia B neonates were achieved on day of life 0 (*i.e.*, the day of birth) by overexpressing FIX Fc fusion protein in the pregnant mice. In addition, an important finding of this study was that the novel form of FIX (FIX Fc protein) was transplacentally transferrable from pregnant mice to their fetuses. Another key finding was that transplacentally transferred FIX in the neonate showed biologically active coagulation activity sufficient to correct the hemophilic phenotype. The therapeutic value of the transplacentally delivered FIX was confirmed by the improved survival rate in the hemophilia B neonates on day of life 0 (the day of birth), as shown in the tail clipping survival assay (Fig. 4D).

The data on the correlation between plasma FIX levels in the mother and newborn mice indicated that 3–7% of the plasma FIX in the mother mice was present in the newborn mice, when the plasma FIX level in the mother mice was more than approximately 600 ng/ml (Fig. 2E). These data suggest that the plasma level of FIX Fc fusion protein in the mother should be maintained at more than 700 ng/ml, which is approximately 14% of the plasma FIX level in healthy subjects, in order to obtain a therapeutic level of plasma FIX in hemophilia B neonates. Gene delivery vehicle-mediated overexpression of FIX Fc fusion protein is a promising strategy to maintain a sufficient level of plasma FIX level for efficient transplacental delivery of FIX to the fetus. Sustained expression of FIX above 20% of the normal level was achieved in the clinical trials of hemophilia B gene therapy using FIX-expressing adeno-associated vectors [26,27]. Protein replacement therapy using recombinant FIX Fc fusion protein is also a promising approach; however, high amounts of recombinant FIX Fc protein should be repeatedly administered to keep high plasma FIX levels for efficient delivery of FIX to fetus.

The half-life of plasma FIX levels in the newborn wild-type mice was approximately 24 h (Fig. 3). Further treatments, including administration of recombinant FIX, are necessary for correction of hemostatic phenotype in hemophilia B after birth. The half-lives of wild-type FIX in the blood of conventional adult mice and hemophilia B mice were 12.3 and 17 h [28,29]. These data suggest that the FIX Fc fusion protein used in this study exhibited higher levels of blood circulation time than wild-type FIX in the neonatal wild-type mice. Fusion of the IgG Fc region to proteins is widely used primarily to improve the half-life of proteins in blood because the interaction between the IgG Fc domain and FcRn on vascular endothelial cells plays a crucial role in the improvement of the blood circulation time by enhancing the recycling pathway [11,30]. These previous findings suggest that fusion of the IgG Fc domain to FIX prolonged the blood circulation time in the newborn mice in this study.

In spite of plasma FIX levels being sufficient for improvement of the hemostatic phenotype in the hemophilia B newborn mice, immune tolerance to human FIX was not found in the mice (Fig. 5), probably because plasma FIX levels in the newborn mice were too low to induce immune tolerance and gradually declined with a half-life of 24 h. We previously demonstrated that when a therapeutic level of human FIX in plasma was maintained for more than 70 days by Ad vector-mediated overexpression of human FIX in 2-day-old neonatal mice, immune tolerance to human FIX was induced [14]. It is necessary to maintain

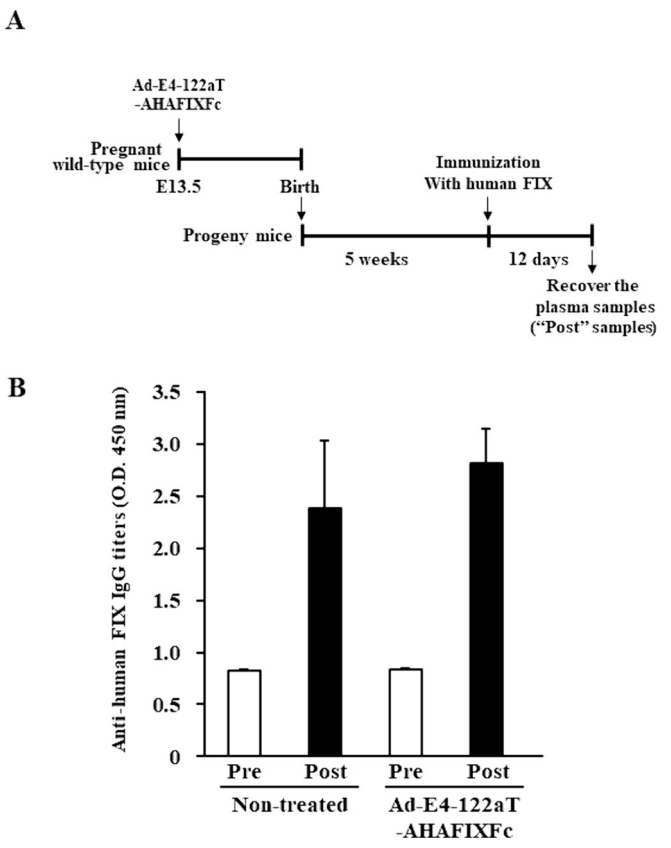

Fig. 5. Serum anti-human FIX antibody titers after immunization with human FIX protein in the offspring of Ad-E4-122aT-AHAFIXFc-treated pregnant mice. (A) The schedule of this experiment. (B) Serum anti-human FIX antibody titers in the offspring of Ad-E4-122aT-AHAFIXFc-treated pregnant wild-type mice. E13.5 pregnant mice were intravenously administered Ad-E4-122aT-AHAFIXFc at a dose of 5.9×10^{11} IFU/kg. Five-week-old mouse offspring were immunized with human FIX protein. Serum samples were collected before immunization ("Pre" samples) and 12 days after immunization ("Post" samples), followed by measurement of anti-human FIX antibody titers. Results are shown as average ± S.D. (n = 3).

sufficient plasma antigen levels for a long period immediately from birth for efficient induction of immune tolerance. Further modification of FIX protein is necessary to show more efficient delivery to neonates and long-term blood circulation in neonates for induction of immune tolerance as well as long-term correction of hemostatic phenotype of hemophilia. On the other hand, Gupta et al. demonstrated that intravenous administration of the Fc domain-fused A2 and C2 domains of FVIII in pregnant mice at E16, E17, and E18 induced immune tolerance in their newborn mice [24]. Although the half-life and concentrations of the Fc domain-fused A2 and C2 domains in the newborn mice were unclear in that study, the types of antigens might also be important for the induction of immune tolerance.

An Ad vector containing the four copies of sequences perfectly complementary to a liver-specific miRNA, miR-122a, in the 3′-untranslated region (UTR) of the E4 gene [16]. In this study, Ad-E4-122aT-AHAFIX and Ad-E4-122aT-AHAFIXFc mediated the sustained plasma human FIX levels without severe hepatotoxicity, on the contrary, significant elevation of serum ALT an AST was found in AdRGD-CMVFIXFc-injected mice (Fig. 1B, C). Safety concerns, including hepatotoxicity, are the most important issue, especially for gene therapy during pregnancy. The liver also plays a crucial role during pregnancy. Functions and abilities of the liver during pregnancy are different from those in normal states [31,32]. In addition, the AHA promoter, which mediated liver-specific transgene expression [33], was used for expression of FIX Fc fusion protein, because liver-specific FIX expression is important for induction of immune tolerance to FIX [34,35]. Immune tolerance to human FIX was induced when human FIX was expressed in a liver-specific manner using the AHA promoter, while immune responses of human FIX were efficiently induced when human FIX was ubiquitously expressed [35]. FIX Fc fusion protein was efficiently and sustainedly expressed in the liver of pregnant mice after Ad vector administration.

Nakanishi et al. previously demonstrated that an Ad vector containing the RGD peptide in the HI loop efficiently transduced the placenta following intravenous administration in pregnant mice [36]. In this study, detectable levels of FIX in the plasma of neonatal wild-type mice were not found following intravenous administration of AdRGD-CMVFIX in the pregnant wild-type mice (Supplemental Fig. 3), suggesting that Ad vector-mediated over-expression of wild-type FIX in the placenta did not lead to the delivery of FIX to the neonatal mice.

AAV vectors are clinically used for liver-directed gene therapy of hemophilia due to the superior transduction profiles [37]; however, previous studies suggested the serious concerns about AAV vector-mediated *in utero* gene therapy [38,39]. The trophoblast dysfunction occurred following wild-type AAV infection, and placental AAV infection was associated with preeclampsia [38]. Adverse reproductive outcomes in early pregnancy of wild-type AAV-infected women were also reported [39]. In addition, hepatocellular carcinoma was found following neonatal administration of AAV vectors due to the insertional mutagenesis [40,41].

In summary, we demonstrated that the hemostatic phenotype of hemophilia B newborn mice was significantly improved by transplacental transfer of FIX fused with the IgG Fc domain, which was overexpressed by an improved Ad vector, from the pregnant mice to their fetuses. These findings provide important clues for the treatment of hemophiliac fetuses *in utero*.

CRediT authorship contribution statement

Fuminori Sakurai: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Shunsuke Iizuka:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tomohito Tsukamoto:** Methodology, Investigation. **Aoi Shiota:** Methodology, Investigation. **Kahori Shimizu:** Investigation, Methodology. **Kazuo Ohashi:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Hiroyuki Mizuguchi:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

Data availability

The datasets analyzed during this study are available from the corresponding author on reasonable request.

Acknowledgements

We thank Ikuho Ishigami, Miu Hayashi, Yukiko Ueyama-Toba, Eiko Sakai, Kazuo Takayama (Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan) and Kohei Tatsumi (Kinki University, Osaka Japan) for their support. The human FIX gene was kindly provided by Tomohiko Yamazaki (World Premier International Research Center for International Center for Materials Nanoarchitectonics, National Institute for Materials Science, Tsukuba, Japan). This study was supported by a Grant-in-Aid for Scientific Research (A) (20H00664, 23H00552), Young Scientists (A) (23689010) from the Ministry of Education, Culture, Sports, Sciences, and Technology of Japan, the Platform Project for Supporting Drug Discovery and Life Science Research

(Basis for Supporting Innovative Drug Discovery and Life Science Research [BINDS]) from the Japanese Agency for Medical Research and Development (AMED) (JP24ama121052, JP24ama121054), and a grant from the Sumitomo Electric Industries Group CSR foundation and Bayer Hemophilia Award Program. Shunsuke Iizuka, Aoi Shiota, and Tomohito Tsukamoto were supported by a Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fellows.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2024.08.022.

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