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RESEARCH ARTICLE

Long-term correction of hemorrhagic diathesis in hemophilia A mice by an AAV-delivered hybrid FVIII composed of the human heavy chain and the rat light chain

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Abstract **Conventional therapies for hemophilia A (HA) are prophylactic or on-demand intravenous FVIII infusions. However, they are expensive and inconvenient to perform. Thus, better strategies for HA treatment must be developed. In this study, a recombinant FVIII cDNA encoding a human/rat hybrid FVIII with an enhanced procoagulant potential for adeno-associated virus (AAV)-delivered gene therapy was developed. Plasmids containing human FVIII heavy chain (hHC), human light chain (hLC), and rat light chain (rLC) were transfected into cells and hydrodynamically injected into HA mice. Purified AAV viruses were intravenously injected into HA mice at two doses. Results showed that the hHC + rLC protein had a higher activity than the hHC + hLC protein at comparable expression levels. The specific activity of hHC + rLC was about 4- to 8-fold higher than that of their counterparts. Hydrodynamic injection experiments obtained consistent results. Notably, the HA mice undergoing the AAV-delivered hHC + rLC treatment exhibited a visibly higher activity than those treated with hHC + hLC, and the therapeutic effects lasted for up to 40 weeks. In conclusion, the application of the hybrid FVIII (hHC + rLC) via an AAV-delivered gene therapy substantially improved the hemorrhagic diathesis of the HA mice. These data might be of help to the development of optimized FVIII expression cassette for HA gene therapy.**

Keywords hemophilia A; adeno-associated virus (AAV); human/rat hybrid factor VIII; gene therapy; dual chain strategy

Introduction

Hemophilia A (HA) is an X-linked hemorrhagic disorder characterized by deficiencies in factor VIII (*FVIII*). Patients with severe HA (FVIII:C $< 1\%$) often experience spontaneous bleeding, and the incidence of HA is 1 in 5000 in males [1,2]. The currently available treatment for HA is either on-demand or prophylactic infusion of FVIII concentrate [3]. To date, HA remains incurable. The shortcomings of frequent FVIII infusions are the high expense of treatment, inconvenience in performing the procedure, and the development of inhibitors to FVIII [4].

Gene therapy has the potential to overcome some of the shortcomings of replacement therapy, making it an ideal treatment modality. Clinical application of a viral vector for gene therapy requires sustained therapeutic effects with no apparent vector-related toxicities [5,6]. Adeno-associated virus (AAV) vectors have gained prominence owing to their nonpathogenic nature and low immunogenicity [7,8]. Studies demonstrated that AAV-carrying codon-optimized FIX and FVIII can successfully treat patients with hemophilia B (HB) [9] and HA via gene therapy [10]. Despite these advances, novel FVIII expression cassettes with high-specific activity are still highly anticipated.

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AAV has shown promise for gene delivery into post-mitotic tissues [7]. The development of AAV-mediated gene therapy for HB has been quicker than that for HA because of AAV packaging restrictions (4.7 kb) and the large size of *FVIII* [11,12]. The FVIII protein is a heterodimer with a heavy chain (HC) and a light chain (LC). The full size of *FVIII* cDNA is 7.0 kb and that of the B domain-deleted *FVIII* (*FVIII*-BDD) is 4.3 kb. Although the size of *FVIII*-BDD is ~ 4.3 kb, it is close to the package limit, which leaves little space for some indispensable regulatory elements for AAV-efficient package [12,13]. Splitting FVIII into HC and LC for dual AAV vector delivery is an effective approach [14,15].

The crucial issues of gene therapy for HA are increasing specific FVIII activity and decreasing the dose of AAV vectors [16]. Codon-optimized FVIII and some variants exhibit enhanced coagulation activity [17–20]. FVIII from some other species, such as porcine and canine, displays a higher efficiency in expression or secretion than human FVIII (hFVIII). Porcine FVIII (pFVIII) can secrete more efficiently than hFVIII by 10- to 100-fold [21,22], and canine FVIII (cFVIII) has better activity by three fold than hFVIII-BDD [23,24]. The hybrid human/porcine FVIII (pHC + hLC) displays an enhanced coagulant activity than hFVIII [25], which opens an avenue to construct an interspecies hybrid FVIII. A recent study revealed that rat FVIII (rFVIII) has a higher activity than hFVIII by 2- to 7-fold [26]. Considering that the activity of rFVIII and hybrid FVIII is higher than that of hFVIII, we speculate that the hybrid human/rat FVIII can exhibit elevated FVIII coagulant activity.

Materials and methods

FVIII expression plasmids

cDNAs encoding human FVIII heavy chain (hHC), human light chain (hLC), and rat light chain (rLC) were cloned into AAV vectors with a CB (ubiquitous expression) or an ApoE-hAAT (liver-specific expression) promoter. The AAV vectors were gifts from Dr. Weidong Xiao, whereas hFVIII was from Dr. Guowei Zhang. rLC was synthesized by BioSune Biotechnology Co. Ltd. (Shanghai, China) on the basis of sequences from NCBI (NM_183331.1) [26]. Plasmids with the CB promoter were expressed in 293T cells, and those with the ApoE-hAAT promoter were transfected in HepG2 cells. For hydrodynamic injection and AAV production, the ApoE-hAAT promoter was employed.

rAAV production

Serotype 2-pseudotype 8 vectors were produced by triple plasmid co-transfection [16,27]. Serotype 8 (AAV8) helper plasmid containing serotype 2 (AAV2) rep (rep2) and AAV8 cap (cap8) genes, adenovirus helper plasmid, and AAV FVIII expression plasmids were co-transfected into 293T cells at a ratio of 1:1:1. The transfected cells were harvested 3 days later. AAV was purified by two rounds of cesium chloride gradient ultracentrifugation [28,29]. The dialysis was followed with 5% D-sorbitol in PBS. Vector purity and genome titer were estimated by silver staining and quantitative real-time PCR (qPCR), respectively. The resultant AAV viruses were stored at −80 °C.

Quantitative real-time PCR

First, 10 μL of the vectors were put in a 20 μL system containing 1× DNA digestion buffer (40 mmol/L Tris-HCl, pH 8.0, 10 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$) and 20 U DNase I and then incubated at 37 °C for 30 min. Afterward, the reaction was stopped by adding EGTA to a final concentration of 2 mmol/L. Subsequently, 10 μL of the mixture was further digested in protease K digestion buffer (10 mmol/L Tris, 2 mmol/L EDTA, 0.5% SDS, 1 mg/mL protease K) at 55 °C for 90 min. The digested rAAV vector was diluted 1000× and used as the template for qPCR in a 20 μL reaction system by using PerfectStart™ Green qPCR SuperMix (TransGen Biotech, China). A standard curve was built using 10-fold serial dilutions of a linearized transgene plasmid as the templates. Vector titers were determined via the ΔΔCt method according to the standard curve. The following primers were used: hHC-forward: 5-CTGAAATGGATG-TGGTCAGG-3; hHC-reverse: 5-AGTCCCAGTCCTC-CTCTTCA-3; hLC-forward: 5-CCAGATGGAAGATC-CCACTT-3; hLC-reverse: 5-GCTGAGCAGATACCATC-GAA-3; rLC forward: 5-GCAGAGTATGACGAC-GATGC-3; rLC reverse: 5-GTAGTCCCAAAGCT-CCTCCA-3.

Culture and transfection of 293T and HepG2 cells

293T and HepG2 cells were obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), penicillin (100 U/mL), and streptomycin (Invitrogen, Carlsbad, CA, USA) (100 μg/mL). Polyjet (Signagen, Rockville, MD, USA) was used for transfection following the manufacturer's instructions. After transfection, the cells were cultured for 8–12 h in DMEM with 10% FBS to minimize cell death. The DMEM was then replaced with F10 medium (HyClone, Logan, UT, USA) with 2% inactivated FBS for another 48 h before the medium was collected.

Animal procedures

HA mice with exons 16–19 of the mouse *F8* gene-targeted knock out [30] and bearing the S129 genetic background were introduced from Shanghai Research Center for Model Organisms. In this study, 6- to 8-week-old mice were used. All mice were housed in a specific pathogen-free environment with constant temperature at 21 ± 1 °C, constant humidity at 55% ± 10%, and a 12 h light/12 h dark cycle with free access to food and water. All experimental procedures were approved by the Studies Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. All institutional and national guidelines for the care and use of laboratory animals were followed.

For hydrodynamic injection, 150 μg of the plasmids dissolved in 2.0 mL saline was injected into the HA mice via the lateral tail vein within 10 s. Bleeding rescue was evaluated by half-clipping of the mice's tails at 1.5 mm diameter 72 h after the hydrodynamic injection. Following the method of a previous study [31], at 6 h after the tails were clipped, blood was collected by eye-bleeding from the mice. Residual hemoglobin (Hb) concentration was measured using an animal blood routine detector (Coulter AcT diff analyzer, Beckman, Coulter, USA).

For AAV-delivered FVIII (hHC + rLC and hHC + hLC) gene therapy, the HA mice were randomly divided into five groups: control group (the mice were injected with 200 μL saline only), hHC + rLC intermediate dose group (the mice were injected with the purified AAV viruses carrying hHC and rLC at a ratio of 1:1 at a dose of 8×10^{12} vg/kg), hHC + hLC intermediate dose group (the mice were injected with the purified AAV viruses carrying hHC and hLC at a ratio of 1:1 at a dose of 8×10^{12} vg/kg), hHC + rLC high dose group (the AAV viruses were the same as those of hHC + rLC intermediate group, and the dose was 2×10^{13} vg/kg), hHC + hLC high dose group (the AAV viruses were the same as those of the hHC + hLC intermediate group, and the dose was 2×10^{13} vg/kg). The aforementioned viruses were diluted in 200 μL saline and injected into the HA mice via the tail vein. Citrated blood was collected every 2 weeks post AAV virus administration. Plasma was harvested by centrifuging the blood and stored at −80 °C. The long-term efficacy of gene therapy was observed up to 40 weeks.

Quantitative analysis of FVIII activities and antigen

FVIII clotting assay (FVIII:C) was measured via a one-stage method. Test samples and serially diluted standards were diluted in activated partial thromboplastin time (aPTT) assay buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 1% BSA, pH 7.4). Afterward, 25 μL aPTT reagents (STAGO, Diagnostica Stago S.A.S., France), 25 μL FVIII-deficient plasma (STAGO, Diagnostica Stago S.A.S., France), and 25 μL of the test samples or standards were mixed and incubated for 3 min at 37 °C. Subsequently, 25 μL of $CaCl_2$ (25 mmol/L) was added, and the time of clot formation was measured using a STAGO semi coagulation analyzer (STAGO, Diagnostica Stago S.A.S., France). Normal human plasma (NHP, the pool of human plasma from ~20 normal individuals) was used as the standard. FVIII activity was calibrated by NHP, and the activity of NHP was defined as 100%.

FVIII antigen was determined via enzyme-linked immunosorbent assay (ELISA). The antibody of hHC was used to compare the difference between hybrid FVIII and hFVIII. Nunc Maxisorp plates were coated with 1 μg/mL capture antibody GMA-8016 (Green Mountain Antibodies, Burlington, VT, USA) in a coating buffer (0.1 mol/L sodium bicarbonate and carbonate, pH 9.6) and incubated overnight. A blocking buffer (0.13 mol/L NaCl, 2.8 mmol/L KCl, 3.2 mmol/L Na_2HPO_4, 1.4 mmol/L KH_2PO_4, 0.05% Tween 20, pH 8.4) was then added and incubated for 1 h. Samples and standards were diluted in 1× assay buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 1% BSA, pH 7.4) and then added to plates at 100 μL/well for incubation for 2 h. The plates were washed using PBST for three to five times, and then the detection antibody of biotinylated GMA-8015 (1 μg/mL) (GMA-8015, Green Mountain Antibodies, Burlington, VT, USA) was added. Biotinylated GMA-8015 was prepared using a biotin conjugation kit (Abcam, Cambridge, USA). After washing, horseradish peroxidase streptavidin antibody (100 μL/well, 0.5 μg/mL) (Vector Laboratories, Burlingame, USA) was added. After a final wash, 100 μL of 1× TMB substrate solution (SureBlue TMB 1-component microwell peroxidase) was added. The reaction was stopped by adding 50 μL of 0.5 mol/L H_2SO_4, and the absorbance values were read at 450 nm by using a spectrophotometer (NanoQuant, infinite M200, TECAN, Austria). The amount of FVIII antigen was calculated on the basis of NHP. The initial activity of NHP was defined as one unit that was equivalent to 0.2 μg.

Western blots

The media of 293T and HepG2 cells were collected and centrifuged for three- to five-fold concentration by Amicon® Ultra 3K centrifugal filter devices (Millipore, USA) at 4000 rpm at 4 °C for about 1–2 h. The samples were separated as two parts: one for reduced state, and the other for nonreduced state. For the reduced state, the sample was treated with 100 mmol/L DTT for 30 min and then boiled at 100 °C for 10 min in 2× SDS loading buffer with 100 mmol/L DTT. For the nonreduced state, the sample was boiled in loading buffer without a reducing regent. Western blot (WB) was performed following the method of a previous study [23]. The proteins were separated by 8% SDS-PAGE and then transferred to

polyvinylidene difluoride membrane (GE Healthcare, USA). The membrane was blocked in TBST containing 5% skim milk powder and then incubated with a polyclonal sheep anti-human FVIII antibody (1 μg/mL, Affinity Biologicals, USA). After washing in TBST, the membrane was incubated with HRP-conjugated donkey anti-sheep IgG (H&L) (0.5 μg/mL, Santa Cruz Biotechnology, USA) at room temperature for 1 h. The aforementioned antibodies were diluted in TBST containing 5% bovine serum albumin (BSA). The membrane was visualized by chemiluminescent HRP substrate (Millipore, USA) and imaged using ImageQuant LAS-4000 (FujiFilm, Tokyo, Japan).

Thromboelastography assay

Fresh citrated blood (~500 μL) was collected at 54 h after hydrodynamic injection and immediately used for thromboelastography (TEG) assay within 2 h. Kaolin-activated TEG reagents and cuvettes were purchased from Haemonetics (Heamoscope Corporation, Niles, USA). TEG assay was performed in a TEG 5000 device according to the manufacturer's instructions as described in a previous study [26]. The parameter of "reaction time" represents clot formation time.

ELISA for alanine aminotransferase and aspartate aminotransferase

Mouse alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected using an ALT/AST ELISA Kit (Suer, Shanghai, China).

Statistical analysis

One-tailed Student's *t*-test and one-way ANOVA with Bonferroni's multiple comparison were performed. Analysis was conducted in GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data are displayed as mean ± SD. P values of < 0.05 were considered statistically significant.

Results

Hybrid FVIII exhibited an increased activity in 293T cells

A previous study reported that the activity of rFVIII is higher than that of hFVIII, and it suggested that rLC might contribute to the enhanced activity of rFVIII [26]. On the basis of this supposition, a hybrid human/rat FVIII (hHC + rLC) was generated herein. The plasmids encoding hHC were transfected into 293T cells together with hLC or rLC at a ratio of 1:1 (HC:LC). Results showed that the activity of hHC + rLC was significantly higher than that of hHC + hLC (***$P < 0.001$, Fig. 1A). The expression of FVIII was measured via ELISA to determine whether the elevated coagulation activity of hHC + rLC was due to the increased protein level. Data showed that the expression level of FVIII antigen in the hHC + rLC group was comparable with that in the hHC + hLC group (Fig. 1B). Furthermore, the specific activity (activity versus quantity of FVIII) was calculated on the basis of aPTT (Fig. 1A) and ELISA results (Fig. 1B). Results showed that hHC + rLC exhibited a specific activity of approximately 4.17-fold higher than that of hHC + hLC (***$P < 0.001$, Fig. 1C). Considering that the quantity of FVIII both in the heterodimer (HC + LC) form and the individual form could be detected by ELISA assay by using the hHC antibody, and the coagulation activity of FVIII was only contributed by the heterodimer form, a more accurate quantitative approach was needed. The exact specific activity was assessed by equilibrating the amount of the FVIII heterodimer by comparing the band intensity of the heterodimer in the hHC + hLC and hHC + rLC samples. This result arose because the heterodimers or the individual monomers can readily be identified according to their electrophoretic mobility on nonreduced WB. Results showed that the amount of the FVIII heterodimer was comparable in the hHC + hLC and hHC + rLC groups because the band intensity of heterodimer in hHC + rLC was 1.1-fold higher than that in hHC + hLC (Fig. 1D). The calibrated specific activity of FVIII was thus calculated. hHC + rLC showed a specific activity of about 3.85-fold higher than that of hHC + hLC (***$P < 0.001$, Fig. 1E). These data indicated that the specific activity calculated on the basis of both ELISA and WB under the experimental conditions was basically consistent. ELISA was therefore chosen to estimate the specific activity in this study because of its practicability.

Hybrid FVIII showed an enhanced activity in HepG2 cells

Aside from 293T cells, liver-derived HepG2 cells were also employed to evaluate the delivery efficiency of AAV expression plasmids carrying different FVIII expression cassettes in the liver cells. The hybrid FVIII also exhibited an enhanced activity compared with hFVIII (***$P < 0.001$, Fig. 2A). ELISA and WB results showed that the amount of FVIII was comparable between the two groups (Fig. 2B and 2C), consistent with that in 293T cells. The specific activity of hHC + rLC was approximately 7.95-fold higher than that of hHC + hLC (***$P < 0.001$, Fig. 2D) as calculated on the basis of ELISA and about 7.18-fold higher as calculated on the basis of WB (***$P < 0.001$, Fig. 2E).

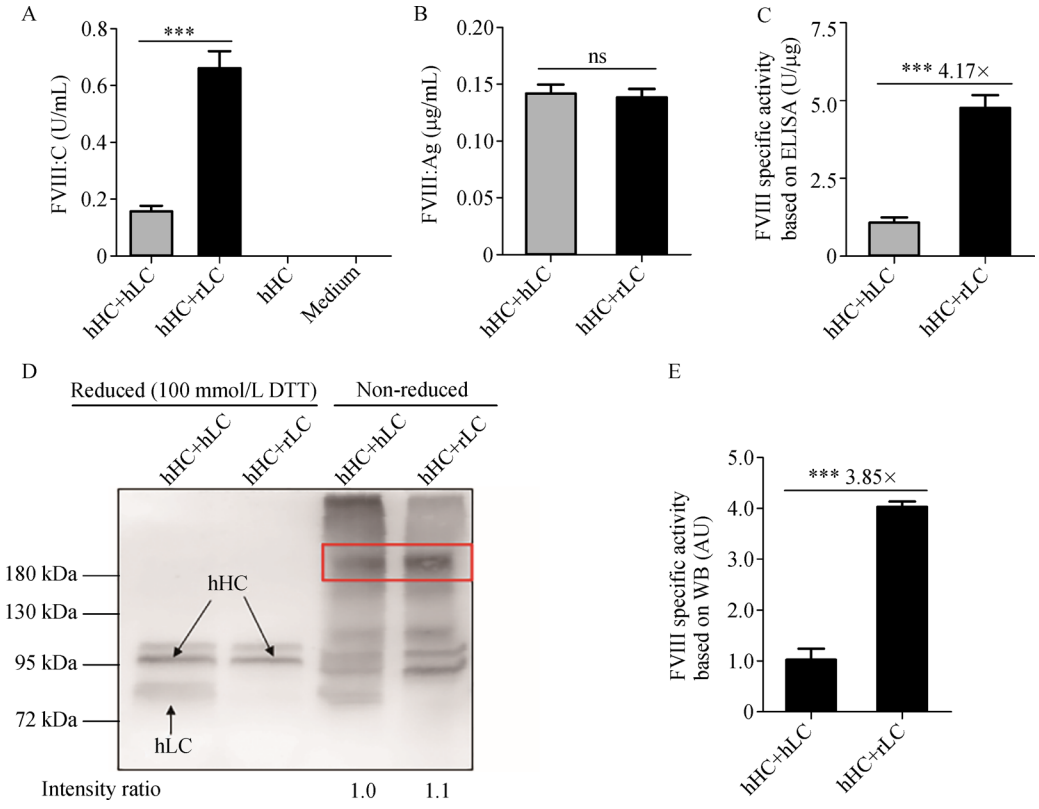

Fig. 1 Activity and antigen expression levels of hHC + hLC and hHC + rLC in transfected 293T cells. (A) FVIII activity of hHC + hLC and hHC + rLC. Expression plasmid of hHC driven by CB promoter was transfected together with hLC or rLC into 293T cells at a ratio of 1:1 (HC:LC), and the total amount of the plasmids was 1 μg/well. At 48 h after transfection, the medium was collected, and FVIII activity was detected via aPTT assay. Normal human plasma (NHP) was used as the standard, and initial activity was defined as one unit. (B) Detection of FVIII antigen by ELISA. FVIII antigen was measured with a specific antibody to hHC. NHP was used as the standard, and one unit was calculated as 0.2 μg. (C) Specific activity of FVIII calculated on the basis of ELISA. The specific activity of FVIII was calculated on the basis of the data from A and B. (D) Detection of FVIII expression by WB. Expression of FVIII in reduced and nonreduced states was detected by WB. The samples used were the transfected cell media of hHC + hLC and hHC + rLC. The intensity ratio of hHC + hLC and hHC + rLC is shown at the bottom. The intensity ratio of hHC + hLC was calibrated as 1.0, and the relative intensity of hHC + rLC to hHC + hLC was 1.1. (E) The specific activity of FVIII calculated on the basis of WB. The specific activity of FVIII was calculated on the basis of the data from A and D. ($n = 3$, $^{***}P < 0.001$.)

Elevated activity of hybrid FVIII was not attributed to its stability

The elevated activity of hybrid FVIII was evaluated to determine if it could be attributed to enhanced stability. FVIII samples from the supernatants of 293T and HepG2 cells were incubated at 37 °C, and the residual coagulation activity was measured by aPTT. Relative FVIII activity could potentially represent the stability of FVIII. As shown in Fig. 3, the relative activity of hHC + rLC was slightly higher than that of hHC + hLC within the first 6 h, but it rapidly declined afterward in both cells. With regard to FVIII from 293T cells, the half-life (the time at which the residual FVIII activity was 50% of the initial point) of hHC + hLC and hHC + rLC was both ~2 h (Fig. 3A). With regard to HepG2 cells, the half-life of hHC + hLC and hHC + rLC was ~2 h and ~4 h, respectively (Fig. 3B). These data indicated that the enhanced FVIII activity of hHC + rLC could not be ascribed to the prolonged half-life and thermal stability.

Hybrid FVIII exhibited an enhanced activity and specific activity in hydrodynamically injected HA mice

Wildtype (WT, normal) mice were employed as the positive control to readily evaluate the coagulation activity of hybrid FVIII *in vivo*. FVIII activities were as follows: 88.2 ± 31.1 for WT mice ($n = 8$), 0 for the HA mice ($n = 3$) injected with saline, 26.8 ± 8.2 for hHC + hLC ($n = 7$), and 71.7 ± 20.3 for hHC + rLC ($n = 7$) (Fig. 4A). The activity of hHC + rLC was approximately 2.7-fold higher than that of hHC + hLC ($^{**}P < 0.01$). The antigen level was comparable between hHC + rLC and hHC + hLC mice (Fig. 4B). The hHC + rLC mice displayed an

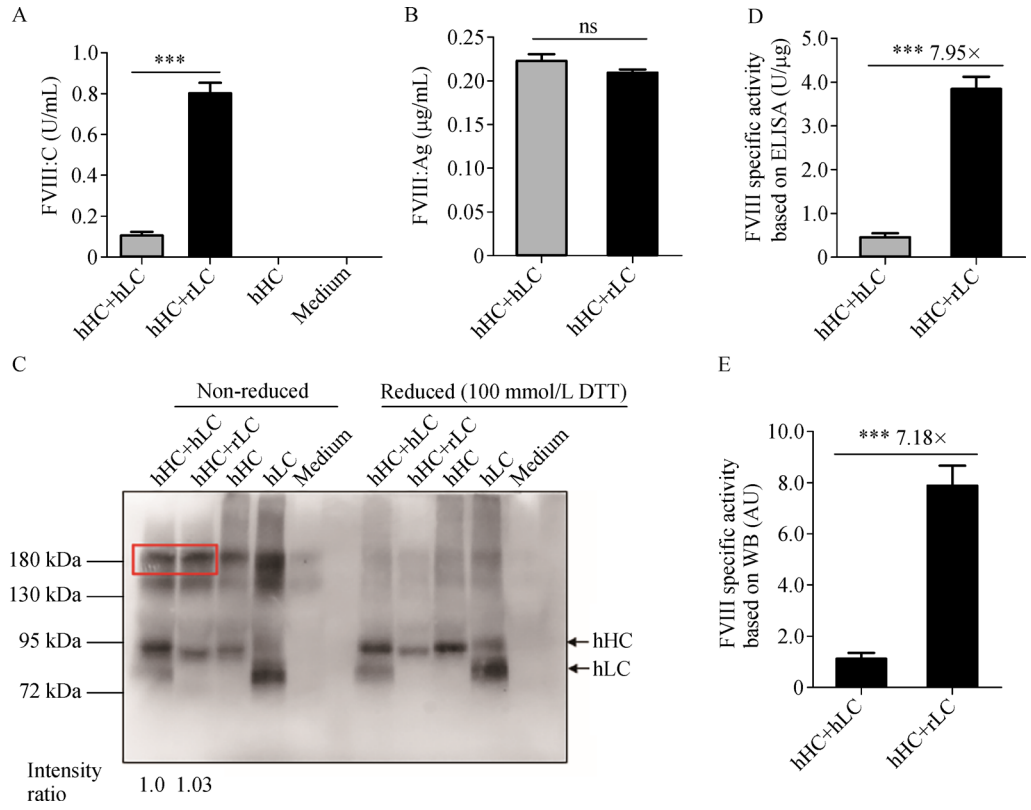

Fig. 2 Activity and antigen expression of hHC + hLC and hHC + rLC in transfected HepG2 cells. (A) Detection of FVIII activity. The expression plasmids of hHC, which were driven by the ApoE-hAAT promoter, were co-transfected into HepG2 cells with LC (hLC and rLC). The total amount of plasmids of each group was 1 μg, and the ratio of HC:LC was 1:1. The activity of FVIII of each group was detected at 48 h post-transfection via aPTT assay. Normal human plasma (NHP) was used as the standard, and initial activity was defined as one unit. (B) Detection of FVIII antigen by ELISA. The FVIII antigens of hHC + hLC and hHC + rLC were monitored by ELISA, and the samples used were the same as those utilized in aPTT assay. NHP was used as the standard, and one unit was calculated as 0.2 μg. (C) Detection of FVIII expression by WB. Expression of FVIII of hHC + hLC and hHC + rLC in reduced and nonreduced states was detected by WB. The intensity ratio of hHC + hLC and hHC + rLC is shown at the bottom. The intensity ratio of hHC + hLC was calibrated as 1.0, and the relative intensity of hHC + rLC to hHC + hLC was 1.03. (D) Specific activity of FVIII calculated on the basis of ELISA. Specific activity of FVIII was calculated on the basis of the data from A and B. (E) Specific activity of FVIII calculated on the basis of WB. Specific activity of FVIII was calculated on the basis of the data from A and D. ($n = 3$, $^{***}P < 0.001$.)

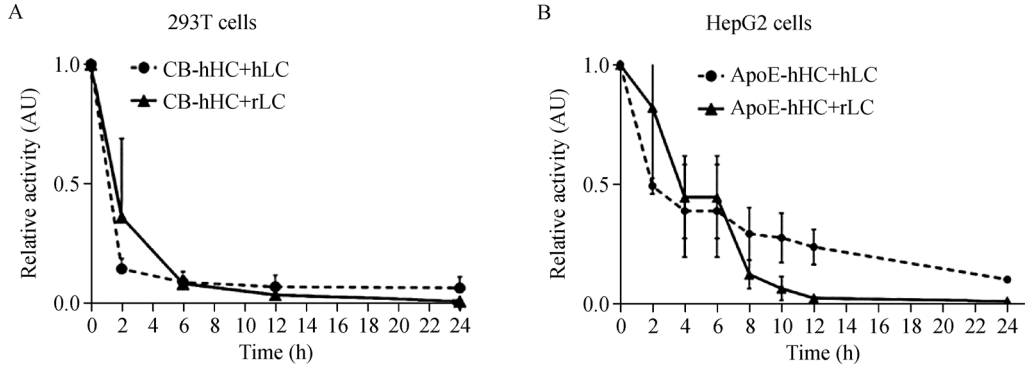

Fig. 3 Detection of the stability of hHC + hLC and hHC + rLC in 293T and HepG2 cells. (A) The sample was the culture medium from the transfected 293T cells. It was incubated at 37 °C for 2, 6, 12, and 24 h. (B) The sample was the culture medium from the transfected HepG2 cells. It was incubated at 37 °C for 2, 4, 6, 8, 10, 12, and 24 h. At the indicated time points, the residual FVIII activities of the samples were measured by aPTT ($n = 3$). Relative activity was calculated according to the activity before incubation at 37 °C, which was defined as 1.0 AU (arbitrary unit).

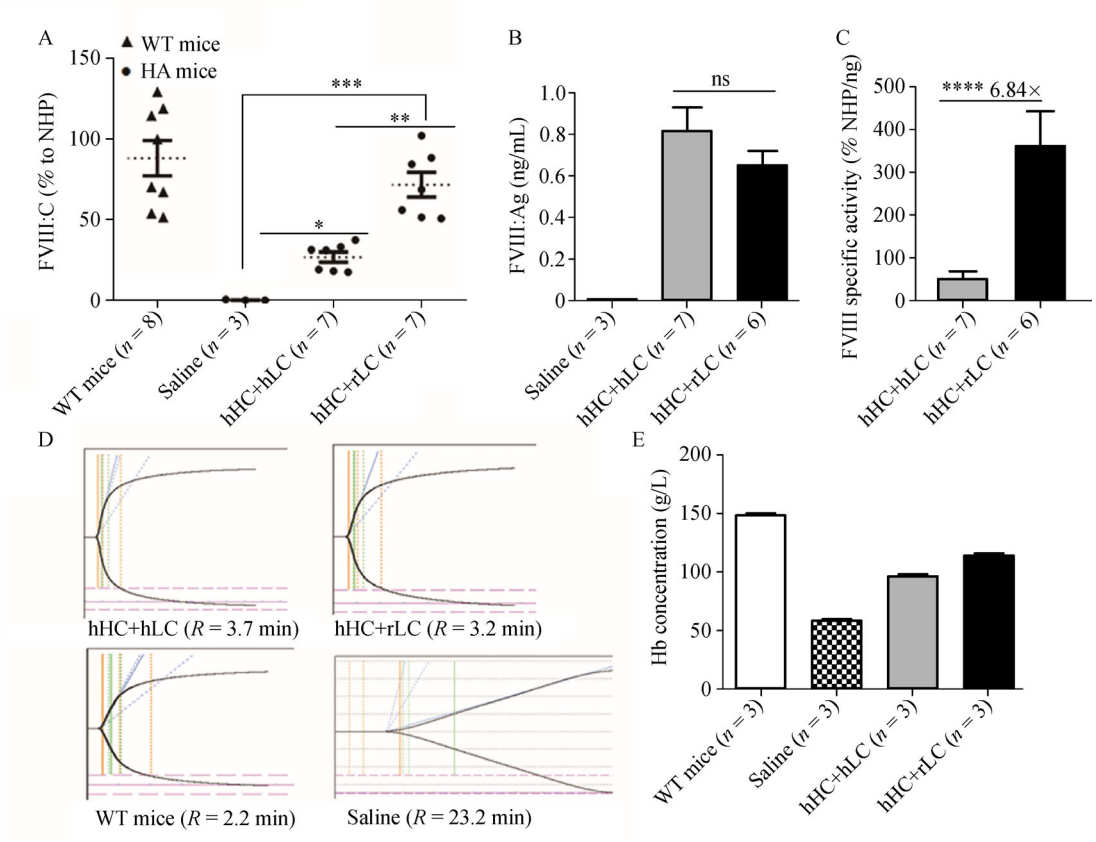

Fig. 4 Detection of FVIII activity, specific activity, TEG, and hemoglobin concentration in the hydrodynamically injected HA mice. (A) Detection of FVIII activity. Expression plasmids for hHC were hydrodynamically injected into HA mice together with hLC and rLC at a ratio of 1:1. A total of 150 μg plasmid diluted in 2 mL saline was injected into each HA mouse through the lateral tail vein. Plasma was collected at 48 h after injection. FVIII activity was detected by aPTT (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Normal human plasma (NHP) was used as the standard. (B) The FVIII antigen was detected using hHC antibody and ELISA. NHP was employed as the standard. (C) Specific FVIII activity was calculated on the basis of the data from A and B (****$P < 0.0001$). (D) Detection of TEG. Fresh citrated blood sample was harvested from the hydrodynamically injected HA mice at 54 h after injection and immediately used for TEG assay. (E) Detection of hemoglobin concentration. Citrated peripheral blood (eye-bleeding) was collected after 6 h from the mouse whose tail was half-clipped. Hb concentration was measured using an animal blood cell counter.

approximately 6.84-fold higher specific activity than the hHC + hLC mice (****$P < 0.0001$, Fig. 4C).

The clot formation of FVIII was measured via TEG assay. The R value is a valuable parameter in evaluating the clotting time from the initial point to when the wave form reaches 2 mm above baseline. The R value was 3.7 min for hHC + hLC mice, 3.2 min for hHC + rLC mice, 2.2 min for WT mice, and 23.2 min for HA mice injected with saline (Fig. 4D). In general, the R value of the two plasmid injection groups was close to that of the WT mice and substantially shorter than that of the HA mice injected with saline. Moreover, the R value of hHC + rLC mice was slightly shorter than that of hHC + hLC mice (Fig. 4D), indicating that both hybrid FVIII and hFVIII could correct the bleeding phenotype.

The effects of phenotypic correction were further estimated. The tail of the mice were half-clipped, and Hb concentration from eye-bleeding was measured. The remaining Hb concentration was used to assess the amount of blood loss. As shown in Fig. 4E, Hb was 95.3 ± 2.1 g/L for hHC + hLC, 113.7 ± 1.5 g/L for hHC + rLC, 147.7 ± 3.1 g/L for WT, and 59 ± 2 g/L for the HA mice injected with saline, indicating that the remaining Hb concentration of hybrid FVIII was higher than that of hFVIII.

AAV-delivered hybrid FVIII exerted long-term therapeutic effects *in vivo*

The exact therapeutic efficacy of hybrid FVIII was determined on the basis of the AAV-delivered FVIII gene therapy. Results showed that the coagulation activity of hybrid FVIII was significantly higher than that of hFVIII in both the intermediate dose (from 2 to 24 weeks) and the

high dose groups (throughout the observation period) (*$P < 0.05$) (Fig. 5A and 5B). The average activity level of hybrid FVIII in the high dose group was significantly higher than that of the intermediate dose group (***$P < 0.001$) (Fig. 5B). The long-term therapeutic effects of AAV-delivered hybrid FVIII lasted up to 38 weeks at the intermediate dose group and approximately 40 weeks at the high dose group.

The expression level and specific activity of FVIII in the high dose group was assessed at the stage when the FVIII activity level was relatively stable (16 and 24 weeks, respectively). The expression level of hybrid FVIII and hFVIII was comparable at the indicated weeks (Fig. 5C), and the specific activity of hybrid FVIII was significantly higher than that of hFVIII ($P < 0.05$): ~2.16-fold at 16 weeks and ~2.29-fold at 24 weeks (Fig. 5D). These results were highly coincident with the data from the *in vitro* assay and hydrodynamic injection assay. Nevertheless, compared with the data at 16 weeks, the specific activity of hybrid FVIII and hFVIII slightly decreased at 24 weeks partially because of the decrease in activity.

Liver function was also assessed by measuring ALT and AST in plasma. A mild, transient increase in ALT was observed in HA mice administered with a high dose of AAV-delivered FVIII (Fig. 6A). Moreover, AST remained within a relatively normal range compared with that before virus injection (Fig. 6B).

Discussion

Owing to the short half-life of FVIII [32], frequent infusions are needed to effectively prevent bleeding occurrence. However, the high cost and some adverse effects of infusions restrict access to the standard care for HA [3,4]. N-glycoPEGylated FVIII (N8-GP) and factor VIII Fc fusion protein (FVIII-Fc) have been recently found to exhibit prolonged half-life [33,34] that can reduce the frequency of FVIII infusion. Another innovative approach is to develop a bispecific antibody that can specifically

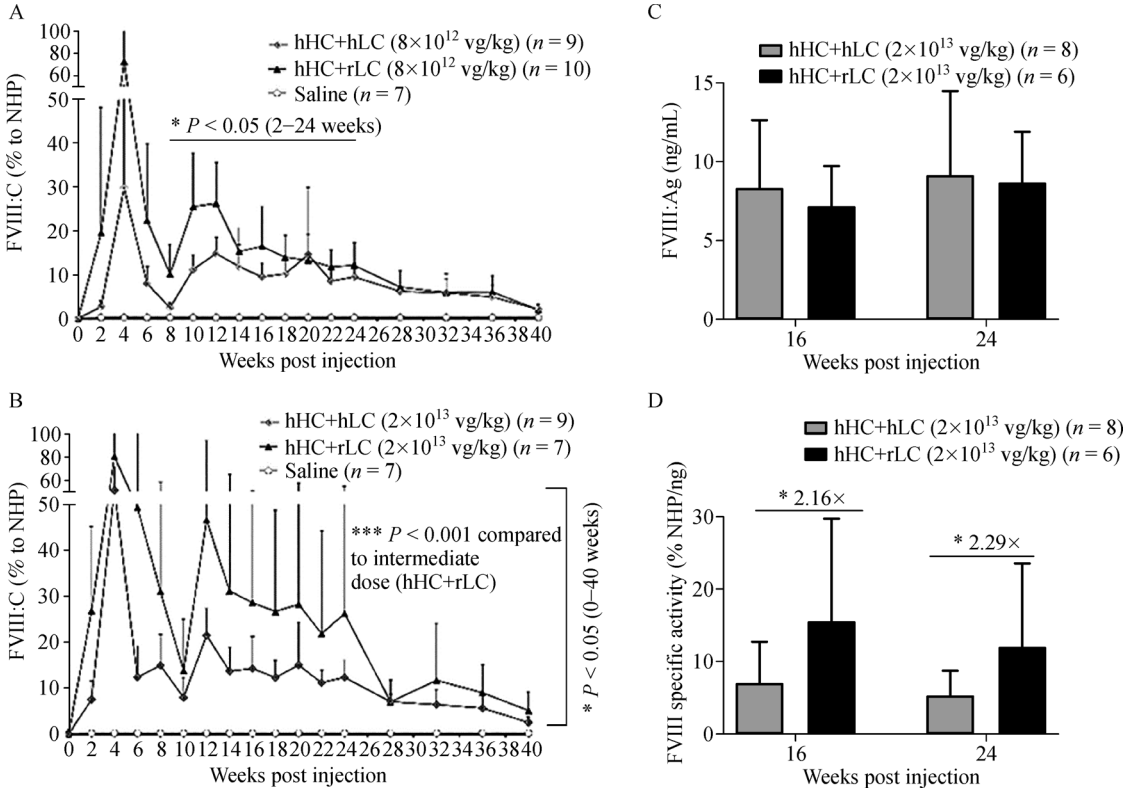

Fig. 5 Detection of the activity and specific activity of hHC + hLC and hHC + rLC in AAV-mediated gene therapy in HA mice *in vivo*. (A) Detection of FVIII activity in the intermediate dose group. AAV viruses delivering hHC together with hLC and rLC at a ratio of 1:1 were injected into HA mice through the lateral tail vein at the intermediate dose group (8×10^{12} vg/kg), and FVIII activity was measured using aPTT assays from 0 to 40 weeks (*$P < 0.05$). Normal human plasma (NHP) was used as the standard. (B) Detection of FVIII activity in the high dose group (2×10^{13} vg/kg). The ratio and the injection method were the same as those in the intermediate dose group. FVIII activity was measured from 0 to 40 weeks (*$P < 0.05$, ***$P < 0.001$). (C) The FVIII antigen of the mice from the high dose group was detected using ELISA at 16 and 24 weeks. hHC antibody was used to detect FVIII expression, and NHP was employed as the standard. (D) Specific FVIII activity at 16 and 24 weeks was calculated on the basis of the data from B and C (*$P < 0.05$).

bind FIX or FIXa on one arm and FX on the other arm to replace FVIII activity *in vivo* [35]. Nevertheless, the clinical efficacy and safety of these approaches must be evaluated. Gene therapy, as an innovative and promising therapeutic strategy for HA, has achieved success in some clinical trials. A recent study showed that infusion of AAV5-hFVIII-SQ achieved a sustained normalization of FVIII activity level over 12 months in patients with HA [10].

AAV-mediated FIX or FVIII transfer to the liver shows great promise as a therapeutic tool for HB and HA gene therapy [36]. AAV8 was applied in some clinical trials and animal models to mediate gene transfer, including FIX and FVIII, to the liver [37]. In the present study, AAV8 was employed for FVIII gene transfer into the HA mice. As expected, the long-term therapeutic effects of AAV8-delivered FVIII were maintained up to 40 weeks in mice *in vivo* (Fig. 5). ApoE-hAAT liver-specific promoter improves the delivery efficiency of some important genes in liver cells [38,39]. Recent clinical trials of hemophilia B gene therapy with a liver-specific promoter (ApoE-hAAT) and a codon-optimized FIX-R338L achieved sustained therapeutic expression of FIX in 10 participants [38]. In the present study, the application of AAV8 capsid and the liver-specific ApoE-hAAT promoter also achieved an efficient FVIII delivery and expression of the hHC + rLC hybrid FVIII into the liver in HA mice (Fig. 5).

A dual-chain strategy was applied in this study to address the package restriction of AAV and the large size of FVIII. The combination of hHC and rLC remarkably enhanced the coagulation activity of FVIII compared with hHC and hLC. Although some studies reported that FVIII activity is higher at HC:LC ratios of 2:1 and 4:1 [14,23,26], the ratio of 1:1 was applied in this study because we were more concerned with the comparison of the activity of hybrid FVIII and hFVIII rather than different ratios.

Enhancing the therapeutic efficacy and reducing the immune response to either AAV capsids or FVIII are the major challenges of HA gene therapy. A recent study reported that a highly specific FIX-R338L activity, a natural gain-of-function mutation of FIX with high specific activity [40], delivered by AAV exhibited a considerably enhanced activity at a lower dose [38], suggesting that seeking gain-of-function mutations of FVIII might also be a promising direction. Unfortunately, unlike FIX, no gain-of-function of FVIII mutant from human was identified, although some other type variants and codon-optimized FVIII exhibit enhanced FVIII activity [17–20]. FVIII from other species, such as porcine [21,22], canine [23,24], and rat [26], displays remarkably elevated FVIII activity. Thus, for hFVIII, FVIII from other species could be considered as a natural mutation pool, and some new mutations bearing higher activity might be readily identified in these species. A previous study proved that the hybrid human/porcine FVIII exhibits a dramatically enhanced activity (44-fold higher) than hFVIII [25]. However, this study did not evaluate *in vivo* effects. On the basis of observations that the FVIII activity of rat is greater than that of human [26] and the hybrid human/porcine FVIII has an enhanced activity when compared to hFVIII [25], a novel hybrid human/rat FVIII was generated herein. As expected, the activity and specific activity of hybrid FVIII were indeed enhanced both *in vitro* (Figs. 1 and 2) and *in vivo* (Figs. 4 and 5). Notably, the coagulation activity of hybrid FVIII was not only higher than that of hFVIII but also higher than that of rFVIII (Fig. 2A), consistent with the results obtained in 293T cells [26]. In fact, the hybrid human/porcine FVIII also displayed a higher activity than hFVIII and pFVIII [25]. These observations implied that hybrid FVIII between species might be a promising direction to optimize FVIII cassettes.

Some studies explored the contribution of domains from HC (A1-A2-B) and LC (A3-C1-C2) to the higher coagulant activity and secretion of FVIII or the inhibition of FVIIIa inactivation. For example, the A1 domain of HC and the A3 domain of LC of pFVIII are responsible for the enhanced secretion of FVIII [22]. The A3 domain of FVIII LC residues 2007–2016 contains an FX interactive site that contributes to the catalytic efficiency of FXase [41]. O'Brien *et al*. [42] indicated that FX, with the overlapping interactive sites and activated protein C (APC) on the A3 domain of FVIII, can reduce the rate of FVIIIa inactivation by inhibiting APC-mediated cleavage at Arg^{336}. Structural studies implicated that the C2 domain plays a predominant role in membrane binding through a combination of electrostatic and hydrophobic interactions [43]. The membrane binding accelerates the activity of the FVIIIa–FIXa complex [44]. In sum, the LC of FVIII contributes to the catalytic efficiency of FXase, protects FVIIIa from being inactivated, and increases the coagulation activity of the FVIIIa–FIXa complex. We also confirmed that rLC combined with hHC showed an elevated coagulation activity compared with hFVIII in an AAV-mediated gene therapy strategy.

Our results demonstrated that the enhanced FVIII activity of hHC + rLC could not be attributed to the prolonged half-life and thermal stability (Fig. 3). Given that the cell culture medium did not have any VWF, the stability of FVIII could not be maintained for a long time. We observed that hHC + rLC activity rapidly decreased after 6 h, which was faster than that of hHC + hLC (Fig. 3). A reasonable explanation is that interspecies FVIII probably has a poorer stability than homogeneous FVIII. Although the stability of hybrid FVIII did not show an advantage compared with hFVIII, the coagulant activity of hHC + rLC was definitely elevated (Figs. 1, 2, 4, and 5), suggesting that the difference in amino acids between rLC and hLC might be responsible for the elevated

procoagulant potential. In fact, homology (~70%) between hLC and rLC was quite high [45]. Therefore, the different amino acids must be determined and the exact key amino acids that contribute to the enhanced activity should be identified.

The long-term therapeutic effects of AAV-delivered hHC + rLC and hHC + hLC could be maintained up to 40 weeks in HA mice. However, a common phenomenon was that the activity of FVIII decreased to the lowest level at 8 or 10 weeks and then gradually increased over time. A similar phenomenon was also observed in previous studies [9,10,26,37]. Studies implicated the fall of activity in FVIII or FIX due to a transient elevation of ALT. When ALT level gradually decreases, the activity of FVIII or FIX accordingly increases [9,10,26,37]. In this study, we also observed a transient elevation of ALT in HA mice (Fig. 6). Therefore, the transient ALT elevation might be a plausible explanation for the fall and subsequent increase in FVIII activity level.

The main goal of this study was to apply the hybrid human/rat FVIII to gene therapy to enhance its coagulatory efficacy. Nevertheless, the development of an inhibitor is a potential risk because of xeno-specific responses. Of course, the overall benefits of particular protocols must be carefully assessed before they are utilized further. Future investigations may determine which amino acid residues are responsible for the enhanced activity by comparing the difference between rat and human sequences. Residues must be selected for less immunodominant ones to facilitate the development of more efficacious protocols for clinical trials.

In summary, we corroborated the existing evidence that the novel hybrid FVIII described herein could potentially enhance FVIII activity and specific activity compared with hFVIII both *in vitro* and *in vivo*. AAV-delivered FVIII could achieve long-term FVIII therapeutic effects on HA mice for up to 40 weeks. The comparable expression level and stability between hHC + rLC and hHC + hLC implied that differences in the sequences and intrinsic properties of rLC and hLC might be responsible for the elevated activity of hybrid FVIII. Developing optimized FVIII expression cassettes on the basis of hybrid FVIII from other species may provide novel perspectives for HA gene therapy.

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Compliance with ethics guidelines

Jianhua Mao, Yun Wang, Wei Zhang, Yan Shen, Guowei Zhang, Wenda Xi, Qiang Wang, Zheng Ruan, Jin Wang, and Xiaodong Xi declare that they have no conflicts of interest. All experimental procedures were approved by the Studies Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. All institutional and national guidelines for the care and use of laboratory animals were followed.

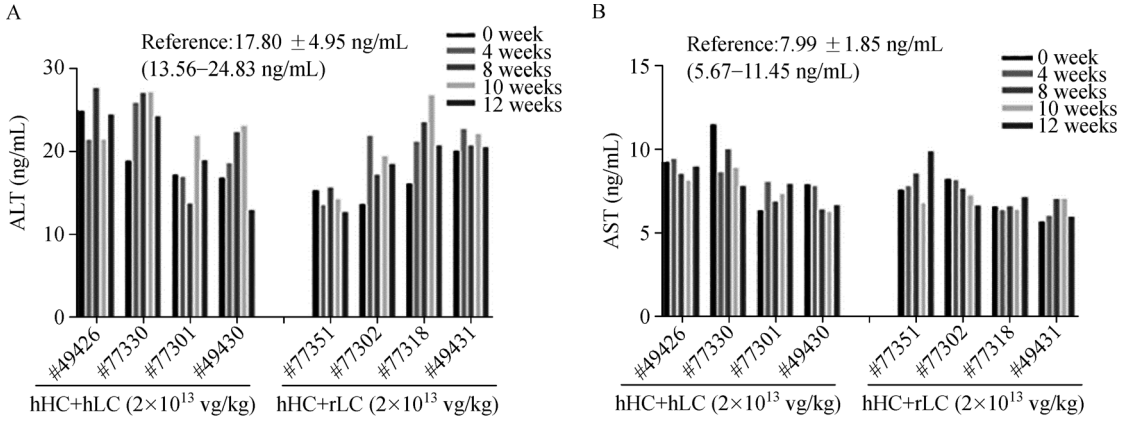

Fig. 6 Detection of liver function. (A) ALT was measured in HA mice administered with a high dose of hHC + hLC and hHC + rLC (n = 4). (B) AST was measured in HA mice administered with a high dose of hHC + hLC and hHC + rLC (n = 4). The reference range of ALT or AST is displayed at the top of the bar chart. The data were from the corresponding HA mice before AAV-delivered FVIII was administered.

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