Evaluation of the biological differences of canine and human factor VIII in gene delivery: implications in human hemophilia treatment

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The canine is the most important large animal model for testing novel hemophilia A (HA) treatment. It is often necessary to use canine factor VIII (cFVIII) gene or protein for the evaluation of HA treatment in the canine model. However, different biological properties between cFVIII and human FVIII (hFVIII) indicated that the development of novel HA treatment may require careful characterization of non-human FVIII. To investigate whether the data obtained using cFVIII can translate to HA treatment in human, we analyzed the differential biological properties of canine heavy chain (cHC) and light chain (cLC) by comparing with human heavy chain (hHC) and light chain (hLC). The secretion of cHC was 5–30-fold higher than hHC, with or without light chains (LCs), cHC + hLC group exhibited ~18-fold increase in coagulation activity compared with hHC + hLC delivery by recombinant adenovirus and adeno-associated viral vectors. Unlike hHC, the secretion of cHC was independent of LCs. cLC improves the specific activity of FVIII by two- to threefold compared with hLC. Moreover, the cLC, but not cHC, contributes to the higher stability of cFVIII. Our results suggested that the cFVIII expression results in the canine model should be interpreted with caution as the cHC secreted more efficiently than hHC and cLC exhibited a more active and stable phenotype than hLC.

INTRODUCTION

Hemophilia A (HA) is the most common genetic disorder associated with serious bleeding, which is caused by defects in the gene coding for factor VIII (FVIII) protein. Protein-replacement therapy, using either plasma-derived or recombinant hFVIII, is currently the mainstay for effective treatment of HA. Because of the short half-life of hFVIII, these treatments must be given frequently. Inhibitor development is considered another significant problem in the current management of HA. These shortcomings highlight the need for developing novel HA therapies that are more efficient and longer lasting. Modified hFVIIIs and FVIIIs from other species with a higher efficiency and a longer half-life were proposed to overcome the disadvantages of current treatments. Vector-mediated and cell-based gene therapy is a novel precision medical option bringing treatment beyond replacement therapy, which may offer longer lasting benefit and even permanent cure. Among the viral vectors, recombinant adeno-associated viral vector (rAAV) is one of the most promising candidates for FVIII gene delivery.

Several well-characterized animal models for HA, including mouse, rat, dog, sheep and pig, have been developed from spontaneous mutations or by targeted knockout of the native FVIII genes in the corresponding HA animal models. For example, canine FVIII (cFVIII) is significantly less immunogenic than the hFVIII when delivered to the hemophilic dogs. Most dogs received cFVIII treatments either by protein infusion or gene therapy can be repeatedly treated without developing inhibitory antibodies. In addition to the apparent immunogenic properties, FVIII proteins from different species also exhibited dramatic differences in biological and biochemical properties. It is well known that porcine FVIII (pFVIII) secretes 10–100-fold more efficiently than hFVIII. The A1 domain of the heavy chain (HC) and A3 domain of the light chain (LC) of the pFVIII are responsible for the enhanced secretion of the protein. On the other hand, cFVIII is shown to be 3–7-fold more active than hFVIII. A single R1645H amino change in hFVIII was able to significantly improve a specific activity. The differential glycosylation pattern of the ovine FVIII (oFVIII) seems to account for the nearly twofold higher specific activity than hFVIII. Moreover, pFVIII, cFVIII, oFVIII and murine FVIII (mFVIII) all have an enhanced stability in the A2-domain compared with hFVIII after activation. Efficient FVIII gene delivery using rAAV vectors has generally been demonstrated in the murine and canine models either by single-chain or dual-chain injection. The single-chain rAAV vectors carried an A-domain-deleted FVIII (BDD-FVIII). However, it is difficult to generate recombinant vector at sufficient titer because of the large size of the BDD-FVIII, which is very close to the packaging capacity of rAAV. Moreover, the size of the BDD-FVIII limits the choice of regulatory elements, including

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promoters, enhancers, introns and poly A sequences in the vector. The dual-chain strategy in which one vector carrying the HC and another carrying the LC of FVIII is a promising method for testing the efficiency of rAAV-based HA gene therapy, especially in large animal models. In mouse model, biologically active hFVIII were produced when equal amount of human HC (hHC) and human LC (hLC) were injected. However, lower level of hHC (10- to 100-fold) compared with hLC protein was found in the plasma of treated animals. There is also a ‘chain imbalance’ issue in dual-chain strategy when delivering cFVIII in dogs which showed four to eight times more abundant canine LC (cLC) than the canine HC (cHC). Comparing with hHC and hLC, the cHC and cLC co-delivery showed a moderate imbalance. The reasons for this phenomenon were not clear.

To investigate whether differential biological properties of cFVIII and hFVIII may have complicated efficient rAAV vector delivery and FVIII expression, here we examined/studied the biochemical properties of cHC and cLC. Interestingly, we found that the secretion of cHC was much more efficient (5–30-fold) than that of hHC. Moreover, the specific activity of FVIII based on cLC was 2–3-fold higher than that based on hLC. FVIII with cLC also exhibited better thermal stability than that with hLC. The secretion efficiency of cHC was LC-independent, which is different from hHC. Because of the substantial biological difference between cHC and cLC vs hHC and hLC, one has to be cautious when translating results from the canine model with cFVIII into non-human primate studies or human clinical trials.

RESULTS
Both cHC and cLC contribute to higher coagulation activity of cFVIII in vitro
It is known that cFVIII is biologically more active than hFVIII; however, it remains unclear which part of cFVIII is the key domains for the enhanced activity. We first compared the activity of FVIII formed by hHC+hLC and cHC+cLC. When the HC or LC expression constructs driven by cytomegalovirus promoter was transsected into tissue culture cells individually, no FVIII activity could be detected. In contrast, hHC+hLC co-transfection in HEK 293 cells resulted in FVIII activity of 0.17 ± 0.01 U ml⁻¹ as compared with 1.43 ± 0.08 U ml⁻¹ for cHC+cLC combination under similar conditions as measured by activated partial thromboplastin time assays (Figure 1a). The coagulation activity of cHC+cLC combination was determined to be five times more than that of hHC+hLC combinations in BHK cells (Figure 1b). We further analyzed the activity of cHC+hLC and hHC+cLC combination in vitro. Interestingly, cHC+hLC exhibited 2–3-fold increase in coagulation activity over the hHC+hLC combination. Similarly, the combination of hHC+cLC produced 3–5-fold better activity than the cHC+cLC combination. To confirm the role of HC and LC in coagulation activity in vivo, we compared the activities of the permutation combinations by hydrodynamic injection of their expression constructs in BALB/c HA mice. Reconstituted FVIII activity from cHC+cLC was 11.6 ± 4.6 U ml⁻¹, which is approximately eightfold higher than that of hHC+hLC (Figure 1c). The coagulation activity of cHC+hLC was 3.2-fold higher than that of hHC+hLC. hHC+cLC showed 2.2-fold increased compared with hHC+hLC. These results suggest that both cHC and cLC contribute to higher coagulation activity of cFVIII.

cLC improves the specific activity of FVIII
To discern whether the higher coagulation activity of cHC+cLC combination is due to an increased protein level or higher specific activity, combinations of hHC with cLC or hLC were analyzed side by side (Figure 2). In HEK 293 cells, the hHC antigen level of hHC+cLC combination is approximately twofold higher than that with hHC+hLC. By using hydrodynamic injection in mice, hHC+cLC combination only showed 1.29-fold hHC antigen level as compared with hHC+hLC, which is not statistically different. In contrast, the specific activity of FVIII with a cLC is approximately twofold higher than that with an hLC, both in vitro and in vivo. Similar results were observed with FVIIIIs consisting of chHCs. Taken together, these data indicated the major contribution of cLC in enhancing the FVIII activity is not because of a higher antigen level but more likely because of a higher specific activity.

FVIII with a cLC exhibited better thermal stability
It was previously reported that cFVIII was more stable than hFVIII in in vitro assay.ª We hypothesized that cLC accounts for the
increased stability of cFVIII. To test this hypothesis, we tested FVIIIs formed by different combination of human and canine HCs and LCs at 37 °C for extended amount of time and determined the residual coagulation activity. As presented in Figure 3, the half-life of FVIII containing a cLC was much longer than that with a hLC. In contrast, FVIIIs with a cHC did not show significant improvement in thermal stability. This study suggested that cLC is the primary contributor for higher thermal stability of the corresponding FVIII molecule, which may have contributed to more HC accumulation as shown in Figure 2.

FVIII with a cHC secretes better than that with a hHC
The inefficient secretion of hHC led to chain imbalance, in which the LC is overwhelming secreted more that of the HC. To study if increased coagulation activity in FVIII with a cHC was due to the enhanced secretion, we determined the amount of FVIII secreted with either hHC or cHC. As showed in Figure 2, the FVIII with a cHC showed five- to sevenfold and 16–18-fold higher antigen level than that with a hHC in vitro and in vivo, respectively. In a separate experiment, we determined if the cHC was a limiting factor for the secreted FVIII activity by limiting the amount of hLC plasmid (0.2 μg) for transfection (Figure 4). The resulting FVIII activity and antigen level from various amount of HC plasmid exhibited a dose-dependent response. Although the antigen levels of cHC were much higher than those of hHC, the LC is generally in excess than the HC in this study.

chC secretion is independent of LC
To determine if LC can further increase cHC expression, we measured the expression of FVIII with varying LC plasmids for the transfection (Figure 5). As expected, high expression of hLC not only help in hHC but also hHC × c secretion, a molecule that was previously demonstrated to secrete better than hHC. In contrast, hLC failed to increase the cHC secretion. Similar results was observed when used cLC to replace hLC. Consistent with our previous data, the hHC antigen was barely detectable in cells when it transfected alone. However, a relative high cHC antigen level was detected in the media of cells that only were transfected with cHC, both in HEK 293 and BHK cells (Figure 6). In the absence of an LC, the antigen level of cHC was 20–30-fold higher than hHCHL. Expression of different forms of FVIII from hydrodynamically injected mice further support the hypothesis that the cHC secretion is independent with LCs. As showed in Figure 6, cHC alone got 15 U ml⁻¹ of antigen, which was similar with that of co-injection with cLC or hLC. A western blot experiment was done to further support the conclusion that the secretion of cHC is independent with LCs which showed the cHC alone have high antigen level secretion.

The in vivo differential properties of canine and human FVIII heavy chain and light chain complicate the interpretation of gene delivery results
The aforementioned results showed that FVIII can be secreted efficiently using plasmids transfection and hydrodynamic injection, we sought to apply this successful approach to an animal model
and determine if we can achieve continuous endogenous FVIII expression. To make rAAV vectors, all the HCs and LCs were under the control of a hepatocyte-specific promoter hAAT combined with an ApoE enhancer. Mice were injected rAAV8-HCs and rAAV8-LCs at ratio 4:1 and the total dose was 5 × 10^{11} vg per mouse. Mice received cHC+hLC vectors expressed a mean value of 4.8 ± 2.5 U ml^{-1} at 6 weeks and then kept stable during 8–12 weeks around 3.0 U ml^{-1} (Figure 7). The FVIII activity of cHC+cLC group was about twofold increase compared with that of cHC+hLC group. Comparing with hHC+hLC group, which only had a peak coagulation activity of 0.26 ± 0.16 IU ml^{-1} at 2 weeks, cHC+hLC group exhibited ~18-fold increase. We also injected cHC+LC vectors into CD4 knockout C57BL6/Svj129S HA mice. It showed 20–30-fold increase in coagulation activity compared with hHC+LC group (data not shown).

To assess whether cHC molecule could overcome the chain imbalance issue, we compared the expression levels of HCs and LCs in each group. The antigen levels of HC and LC measured by ELISA were presented in Figure 7. hLC expression level in cHC+hLC and hHC+ hLC groups were 4–7 U/ml at 6 weeks and then kept stable during 8–12 weeks around 3.0 U ml^{-1} (Figure 7). The FVIII activity of cHC+cLC group was about twofold increase compared with that of cHC+LC group. Comparing with hHC+LC group, which only had a peak coagulation activity of 0.26 ± 0.16 IU ml^{-1} at 2 weeks, cHC+LC group exhibited ~18-fold increase. We also injected cHC+LC vectors into CD4 knockout C57BL6/Svj129S HA mice. It showed 20–30-fold increase in coagulation activity compared with hHC+LC group (data not shown).

DISCUSSION

Canine HA is the most important large animal model for evaluating rAAV-based gene therapy. Successfully translating cFVIII regimen in HA dog to human can bring effective HA treatment beyond replacement therapy. However, the large size of cBDD-FVIII makes it difficult to be efficiently packaged into rAAV vectors and thus is hard to provide sufficient amount of vectors for canine HA treatments. The dual-chain strategy was proposed to test the efficiency of rAAV-based gene therapy in large animal models. The dose required to achieve therapeutic levels of FVIII in dogs was comparable for single-chain and dual-chain delivery. 13,21 One of the major problems of the dual-chain strategy is the ‘chain imbalance’ issue. Previous data showed that the levels of hLC were typically 10–100-fold higher than those of hHC. 28,29 In our present study, LC and HC antigen level was in the same order when using cHC+LC dual-chain strategies. Therefore, the improvement of chain imbalance issue by cHC is due to the high expression properties. Here we demonstrated that due to the high expression properties of cHC, the chain imbalance issue can be resolve using cHC to replace the hHC.

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Figure 3. Stability of canine–human hybrid FVIII. HCs and LCs(2:1) were co-transfected into HEK 293 cells using PolyJet. Media were harvested 36 h after transfection and tested FVIII activity by aPTT assay immediately. Then, the samples were incubated at 37 °C. At different time points, the residual coagulation activity of samples were measured by aPTT assay again. (a) The stability of FVIIIs formed by hHC or hHCHL with hHC or cHC. (b) The stability of FVIIIs formed by cHC with hLC or cLC. Standard error is shown (n = 3). aPTT, activated partial thromboplastin time.

Figure 4. The secretion property of cHC and hHC. cHC or hHC were transfected into HEK 293 cells along with hLC or cLC at ratios as indicated using PolyJet. Media were harvested 36 h after transfection. The activity of secreted FVIII was determined by aPTT assay. hHC and cHC antigen were measured by human HC-specific ELISA and cFVIII-EIA kit, respectively. In a and b are shown the FVIII activity and antigen level varied with the amount of HCs (n = 5). aPTT, activated partial thromboplastin time.
We should capitalize the results obtained from cHC+cLC injection, as the chain imbalance issue of cHC+cLC is not as significant as hHC+hLC. Thus, it is important to understand the underlying mechanism that contributes to the improved chain imbalance of cHC+cLC. We previous showed that by increasing the secretion of hHC or involvement of more hHC molecules can significantly ameliorated chain imbalance and increase the FVIII activity. Thus we postulated that the moderate imbalance between cHC and cLC compared with hHC and hLC may be due to the better secretion of cHC. In this study, we demonstrated replacing hHC with cHC increased the coagulation activity by ~18-fold and the overall antigen of LC and HC is similar in vivo (Figure 7). Thus the problem of chain imbalance was solved. The dramatic increase of coagulation activity of cHC is not likely due to the high specific activity, as cFVIII only exhibited three- to sevenfold specific activity relative to hFVIII. It is also not likely due to the prolonged half-life of the antigen, because our in vitro data showed that the clC is primarily response for the stability of cFVIII (Figure 2). The major reason for the increase activity of cHC is more likely to be related to secretion, which was able to secret at a high level without helping of LC (Figures 4–6).

FVIII with cHC and hHC dose not only different in secretion but also in immunogenic properties (data now shown). It is interesting to figure out which domain contributes the difference between cHC and hHC. The cFVIII shares high homology with its human counterpart, and their amino acid sequences have a similarity of 80%. However, close inspection of hFVIII crystal structure (2R7E) and cFVIII model predicted based on hFVIII structure reveals significant difference in HC and relative minor variance in LC (Figure 8). Unlike hHC, cHC is constituted with more beta sheet and a-helix in their secondary structure, whether these differences confers advantage of structural stability as observed in function assay is an interesting subject for future research. The secretion of B domain-deleted pFVIII is significantly greater than of hFVIII. The A1 domain of in porcine HC is associated with the high level expression of pFVIII. Our unpublished results also showed that the secretion of porcine HC was much better than that of hHC. And replacement five amino acids of hHC with corresponding porcine HC amino acids (I86V, A108S, G132K, M147T and L152P) significantly increased hHC secretion. The reasons for the high secretion levels of these cHC and porcine HC are not clear. Future studies would be warranted to investigate the mechanism regulating the HC secretion.

For the dual-chain strategy, most studies have been focused on HCs. In our present study, we showed that modulating the LC can potentially improve the outcome of dual-chain strategy. As shown in Figure 1, clC increased the coagulation activity by four- to fivefold in vitro and threefold in hydrodynamic injection study. By using rAAV delivery, clC also showed two- to threefold increase in coagulation activity compared with hLC. The clC may contribute the high coagulation activity via an elevated specific activity. The differential activity of hLC and clC partly explained the three- to sevenfold specific activity difference between hFVIII and cFVIII. It was reported that the activated form of cFVIII was more stable than activated hFVIII. The amino acid difference at the PACE/furin cleavage site within the B domain may partly contribute this difference, as one amino acid change (R1645H) at this site has a twofold increase in biological activity. Interestingly, the clC of cFVIII also contains 1645H at this site. As shown in Figure 2, the half-life of FVIII that possesses clC is significantly longer than that contains hLC. Our results showed that it was clC but not cHC contributed to the stability of cFVIII. On the basis of the fact that cFVIII secretes predominantly in a single-chain form, whereas the hFVIII predominantly secretes as a heterodimer form, one possibility is that the prolonged half-life of cFVIII could be due to the formation of more single-chain form. Indeed, the R1645H
substitution increased the half-life of FVIII by threefolds. However, in our study, all FVIIIs were formed by a dual chain, and secreted as a heterodimer form. Thus, the enhanced stability of cFVIII may not due to the single-chain formation. The underlying mechanisms for cLC-mediated enhanced stability are not known.

Recent developments in bioengineering of coagulation factors provided a series of novel molecules for HA gene therapy. In addition to cFVIII, FVIII from other species further expanded the source of therapeutic molecules. This progress offers the opportunity to modify the dual-chain strategy using rAAV vectors. However, the different biological properties between hFVIII and FVIII of other species indicated that the development of novel HA treatment requires careful characterization of non-human FVIII. It was reported that the doses led to total correction in HA mice can only achieve partial correction in affected dogs. This observation suggested that the mouse may not be an ideal model for predicting the therapeutic dose of rAAV vectors in the HA dogs. Thus, it is recommended that when translating FVIII treatment from one animal model to another or to human therapy, we should carefully investigate and characterize the physical and biological properties of FVIIIs.

Taken together, we extensively analyzed the differential properties of canine and human factor VIII HC and LC. We discovered that the secretion of cHC was much better than hHC, while the specific activity and stability of cLC was higher than hLC. Unlike hHC, the secretion of cHC was independent of LCs. Our results suggested that the cFVIII expression results in the canine model should be interpreted with caution as the cHC secreted more efficiently than hHC and cLC showed more active and stable than hLC. The current study highlighted a very important and largely overlooked issue when translating HA gene therapy with FVIII from animal model to clinical application.

MATERIALS AND METHODS
FVIII expression plasmids
The plasmids containing hHC, hHC<sub>745</sub>, hLC, cHC and cLC had been used in our and others previous studies. This progress offers the opportunity to modify the dual-chain strategy using rAAV vectors. However, the different biological properties between hFVIII and FVIII of other species indicated that the development of novel HA treatment requires careful characterization of non-human FVIII. It was reported that the doses led to total correction in HA mice can only achieve partial correction in affected dogs. This observation suggested that the mouse may not be an ideal model for predicting the therapeutic dose of rAAV vectors in the HA dogs. Thus, it is recommended that when translating FVIII treatment from one animal model to another or to human therapy, we should carefully investigate and characterize the physical and biological properties of FVIIIs.

Figure 6. The cHC secretion is independent of LCs. (a and b), the antigen levels of cHC and hHC in cell culture medium. One microgram of plasmids expressing cHC, hHC<sub>745</sub> or hHC<sub>HL</sub> were transfected into HEK 293 (a) or BHK (b) cells. hHC and cHC antigen in the media were measured by human HC-specific ELISA and cFVIII-EIA kit, respectively. Standard error is shown (n = 4). (c) Comparison of antigen level of cHC and hHC in vivo. Seventy-five microgram of DNA diluted in 2 ml of saline were administrated to hemophilia A mice (n = 5) by hydrodynamic delivery. Plasma was collected 48 h after plasmid administration. The antigen levels of cHC or hHC were determined by ELISA. (d and e) Western blot results of hHC and cHC secretion, with or without LCs. The media containing HCs was harvested and incubated with anti-FVIII antibodies, GMA-8015 (for hHC) or C-FVIII-E1A-C (for cHC). The complex were enriched with protein AG magnetic beads and eluted with 2 × Laemmle sample buffer. The proteins were separated by 10% SDS–polyacrylamide gel and then transferred to nitrocellulose membrane for western blot using a polyclonal sheep anti-human F8 antibody and C-FVIII-E1A-D. The blot was incubated with IRDye800CW-conjugated donkey anti-sheep IgG (H&L) and detected with an Odyssey Infrared Imaging System. *P < 0.01.

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supplied with 5% CO₂. Transfections were carried out using Polyjet (Signagen, Rockville, MD, USA) following the protocol. After transfection, the cells were grown for 12 h in DMEM with 10% fetal bovine serum to minimize cell death. The cells were then maintained in Ham's/F12 medium (Mediatech, Inc., Manassas, VA, USA) with 2% inactivated fetal bovine serum for 24 h before the cells and medium were collected and the secreted FVIII antigens were analyzed (n = 4, 5 or 6).

rAAV vector preparation
rAAV8 vectors were used in this study and produced by a triple plasmid co-transfection method as described previously. Briefly, AAV8 helper plasmid, adenovirus helper plasmid and rAAV vector plasmid containing HC or LC were co-transfected into HEK293 cells cultured in roller bottles at a ratio of 4:1. The transfected cells and medium were harvested 72 h later. rAAV vectors were purified by two rounds of cesium chloride.

Figure 7. cHC improved the chain balance issue in vivo. HA mice were injected with a total amount of $5 \times 10^{11}$ vg per mouse rAAV8-hHC + rAAV8-hLC (a), rAAV8-hHC+rAAV8-cLC (b), rAAV8-cHC+rAAV8-hLC (c) or rAAV8-cHC+rAAV8-cLC (d) at a ratio of 4:1. Functional FVIII was then assayed and converted from aPTT (y axis). The expression of FVIII hHC (b), cHC (c) and hLC (d) in mouse plasma was measured by ELISA (n = 6). aPTT, activated partial thromboplastin time.

Figure 8. Alignment of cFVIII model (green) with its human counterpart (PDB:2R7E, cyan). The major changes were showed in HC, but only minor differences between LCs. In HC (a) amino acid residues of 81–85, 137–140, 587–595 and 636–641 of human FVIII constitute more organized secondary structure (highlighted in yellow), either as α-helix or β-sheet, while amino acid residues 140–141, 193–199, 311–315 and 331–334 in cFVIII model replaced random loop of hFVIII with helix or β-sheet (red). There are only few mismatch between hFVIII and cFVIII in LC structure (b), with only significant differences at amino acid residues 1955–1957, 1975–1977 (yellow) and 1752–1756 (red).
gradient ultracentrifuge. After the collected rAAV vectors were buffer exchanged extensively against PBS with 5% d-sorbitol, vector purity and genome titer were analyzed by silver staining and quantitative real-time PCR. The final vectors were stored at –80 °C before administration.

Animal procedures
For rAAV transduction in vivo, experiments were carried out with 6- to 8-week-old BALB/c HA, C57BL6/SvJ129S HA and CD4 knockout C57BL6/SvJ129S HA male mice (n = 6). All surgical procedures involving mice were in accordance with established NIH guidelines under approved protocols at the Temple University (ACUP 4142). For rAAV tail vein injection, animals were randomized into four treatment groups. A typical injection volume is 200 μl diluted in saline. Mouse plasma post vector administration was harvested by eye bleeding at regular intervals as described (n = 5). For hydrodynamically injected, 2.0 ml of saline containing 150 μg of HC and PLG plasmids mixed at ratio 1:1 was injected into the Balb/c via the tail vein, over 5–10 s. Blood was collected 48 h after hydrodynamic injection. For coagulation analysis, blood was collected using sodium citrate as an anticoagulant at a final concentration of 0.38% (v/v). The blood samples were then centrifuged at 4 °C for 10 min at 10,000 r.p.m. in a microcentrifuge. The plasma was then collected and stored in –80 °C before FVIII assays.

Quantitative analysis of FVIII antigen and activities
Biologically active FVIII in cell culture media and plasma was measured using the activated partial thromboplastin time assay.10 ReFacto (Wyeth, Philadelphia, PA, USA) was used as the standard. FVIII HC and FVIII LC antigen were determined using ELISA assays.11 All antibodies for hHC (GMA-8015 and GMA-8016-Bio) and hLC (GMA8018 and GMA-8022-Bio) ELISA were purchased from Green Mountain Antibodies (Burlington, VT, USA). For cHC-specific ELISA, the cFVIII-EIA kit (Enzyme Research Laboratories, South Bend, IN, USA) was used according the assay procedure. The media from cHC+cLC co-transfected HEK 293 cells was used to make a standard curve. The initial FVIII activity in the media was measured by activated partial thromboplastin time according the Refecto standard.

Western blots
For detecting secreted HCs in the media, conditioning media was harvested and preserved with addition of protease inhibitor cocktails. The media was incubated with anti-FVIII antibodies overnight at 4 °C. GMA-8015 (Green Mountain Antibodies) was used for capturing hHC, and C-FVIII-E1A-C for cHC (Enzyme Research Laboratories). The complex was enriched with protein AG Magnetic Beads and eluted with 50 μl of 2X Laemmle sample buffer containing 50 m M of fresh dithiothreitol. The blot was incubated with IRDye800CW-conjugated donkey anti-sheep IgG (H&L) at 1:1,000 dilution (Rockland Inc., Gilbertsville, PA, USA) at RT for 1 h in TBST containing 0.25% casein. An Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE, USA) was used to scan the membrane and quantify the signal.

Statistical analyses
Two-tailed Student’s t-tests and one-way analysis of variance with Bonferroni multiple comparison post test were performed. The differences were considered significant when P was <0.05. The analysis was performed using the SPSS 11.0 Software.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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