

# Enhanced Biosynthesis of Coagulation Factor VIII through Diminished Engagement of the Unfolded Protein Response\*

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Human and porcine coagulation factor VIII (fVIII) display a biosynthetic efficiency differential that is being exploited for the development of new protein and gene transfer-based therapies for hemophilia A. The cellular and/or molecular mechanism(s) responsible for this phenomenon have yet to be uncovered, although it has been temporally localized to post-translational biosynthetic steps. The unfolded protein response (UPR) is a cellular adaptation to structurally distinct (e.g. misfolded) or excess protein in the endoplasmic reticulum and is known to be induced by heterologous expression of recombinant human fVIII. Therefore, it is plausible that the biosynthetic differential between human and porcine fVIII results from differential UPR activation. In the current study, UPR induction was examined in the context of ongoing fVIII expression. UPR activation was greater during human fVIII expression when compared with porcine fVIII expression as determined by ER response element (ERSE)-luciferase reporter activity, X-box-binding protein 1 (XBP1) splicing, and immunoglobulin-binding protein (BiP) up-regulation. Immunofluorescence microscopy of fVIII expressing cells revealed that human fVIII was notably absent in the Golgi apparatus, confirming that endoplasmic reticulum to Golgi transport is rate-limiting. In contrast, a significant proportion of porcine fVIII was localized to the Golgi indicating efficient transit through the secretory pathway. Overexpression of BiP, an integral UPR protein, reduced the secretion of human fVIII by 50%, but had no effect on porcine fVIII biosynthesis. In contrast, expression of BiP shRNA increased human fVIII expression levels. The current data support the model of differential engagement of UPR by human and porcine fVIII as a non-traditional mechanism for regulation of gene product biosynthesis.

Factor VIII (fVIII)<sup>2</sup> is a large plasma glycoprotein that circulates at low concentration (1 nM) and plays an integral role in blood coagulation. Deficiency of circulating fVIII activity (1) due to mutations within the F8 locus and defined as hemophilia A, or 2) secondary to other genetic lesions (e.g. VWF, LMAN1, or

MCFD2 mutations), results in a bleeding phenotype in humans that correlates inversely with residual fVIII activity (1, 2). Treatment for persons with severe hemophilia A (<1% normal fVIII activity) consists of prophylactic administration of recombinant human fVIII (rhfVIII) produced using heterologous baby hamster kidney (BHK) or Chinese hamster ovary cell expression systems. However, expression of rhfVIII in these systems is 2 to 3 orders of magnitude lower than that of other similarly sized plasma proteins (3).

Previously, we showed that, despite sharing 83% sequence homology, recombinant porcine fVIII (rpfVIII) is expressed at levels 10–100-fold higher than rhfVIII due to more efficient secretion (4, 5). This expression differential is being utilized for the development of new, improved rfVIII therapeutics and novel gene transfer-based therapies (6–12). Although the exact underlying mechanism for the differential expression is not yet known, it has been temporally and spatially localized to post-translational steps occurring in the endoplasmic reticulum (ER) (5). Studies of the biosynthesis of rhfVIII have shown that interaction with the ER resident chaperone immunoglobulin-binding protein (BiP)/glucose-regulated protein 78 (GRP78) induces up-regulation of the unfolded protein response (UPR) (13, 14).

UPR is a coordinated cellular mechanism designed to regulate the build-up and flow of proteins in the ER and sometimes is referred to as the “quality control pathway” (15). Several mechanisms, including the binding of BiP to misfolded proteins in the ER as well as recombinant protein overexpression, have been shown to induce UPR (13, 16). UPR is primarily composed of three potentially redundant signaling arms involving as key players inositol-requiring enzyme-1, protein kinase RNA-like ER kinase, and activating transcription factor 6. Several key markers of UPR induction have been identified, including increased levels of BiP mRNA, up-regulation of ER stress elements, and splicing of XBP1 mRNA (13, 14, 18, 20, 21). In the current study, we investigated the induction of these key UPR elements during heterologous expression of rhfVIII and rpfVIII. We then intervened by altering the endogenous levels of BiP and the spliced isoform of XBP1 (XBP1s) in high expressing clones and report the effects on rfVIII biosynthesis.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Transduction**—BHK cells were maintained in complete DMEM/F-12 supplemented with L-glutamine and 15 mM HEPES (Invitrogen), 10% FBS (Atlanta Biologicals), and 1% penicillin-streptomycin solution (HyClone) (100 units/ml each) at 37 °C with 5% CO<sub>2</sub>. Transductions with lentiviral par-

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<sup>2</sup> The abbreviations used are: fVIII, factor VIII; BHK, baby hamster kidney; ER, endoplasmic reticulum; BiP, immunoglobulin-binding protein; GRP78, glucose-regulated protein 78; UPR, unfolded protein response; XBP1s, spliced isoform of XBP1; m.o.i., multiplicity of infection; rh, recombinant human; rp, recombinant porcine.

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ticles at various multiplicities of infection (m.o.i.s) were performed by incubating 200,000 cells/well plated on Cellbind® 6-well plates (Corning) with viral-based vectors in a final volume of 1 ml of complete DMEM/F-12 supplemented with 8  $\mu\text{g/ml}$  of Polybrene (Sigma). Twenty-four hours post-transduction, vector-containing medium was replaced with fresh complete DMEM/F-12 and transduced cells were expanded for analysis.

**Stable Expression of Recombinant FVIII**—BHK-M cells stably expressing rhfVIII and rpfVIII were generated as previously described (4).

**Measurement of FVIII Activity**—For all fVIII activity measurements, cells were cultured in AIM V media (Invitrogen) for 24 h before assaying fVIII activity. FVIII activity present in the conditioned medium was measured by activated partial thromboplastin reagent-based one-stage coagulation assay in a ST art Coagulation Instrument (Diagnostica Stago) using human fVIII-deficient plasma as the substrate as previously described (4).

**Signal ERSE Reporter Kit**—Thirty thousand naive, rhfVIII- and rpfVIII-expressing BHK cells were plated in a 96-well plate. After 24 h, the cells were transfected with 100 ng of plasmid encoding firefly luciferase under the transcriptional control of an ERSE inducible promoter and 100 ng of plasmid encoding *Renilla* luciferase under the transcriptional control of a constitutively expressing cytomegalovirus promoter (SABiosciences) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The medium was changed 24 and 48 h post-transfection, thapsigargin (Sigma) was added to wells containing naive BHK cells at a concentration of 500 nM. Seventy-two hours post-transfection, the cells were washed once in phosphate-buffered saline (PBS) (Mediatech) and assayed using the Dual Luciferase Reporter System (DLR) (Promega). The DLR Assay was performed using the DLR injector program on a Veritas Luminometer (Turner Biosystems).

**Immunofluorescence Imaging**—Twenty-five thousand cells per well were plated on collagen-coated culture slides (BD Biosciences) in 800  $\mu\text{l}$  of DMEM/F-12, 10% FBS, penicillin/streptomycin and grown overnight. The following day, the cells were rinsed once with 200  $\mu\text{l}$  of pre-warmed PBS. The cells were fixed for 15 min in 3.7% pre-warmed formaldehyde (Sigma) in PBS at room temperature. The cells were then rinsed 2 times with PBS. Next, the cells were permeabilized by washing 4 times for 5 min each with 200  $\mu\text{l}$  of 0.1% Triton X-100 (Roche Applied Science) at room temperature with gentle shaking. The cells then were rinsed with 200  $\mu\text{l}$  of PBS. Seventy microliters of Image-It FX Signal Enhancer (Invitrogen) was added to each well and incubated for 30 min at room temperature with gentle shaking. Next, the cells were rinsed with 200  $\mu\text{l}$  of PBS. The cells were blocked for 1 h in Rockland Near IR blocking buffer (Rockland Immunochemicals) at room temperature with gentle shaking. Wheat germ agglutinin 350 (Invitrogen) was then added to the cells at a final concentration of 50  $\mu\text{g/ml}$  in PBS and incubated for 10 min at room temperature with gentle shaking. The cells then were rinsed twice with PBS. Human-porcine cross-reactive murine anti-FVIII immunoglobulin G (IgG) 512G (kindly provided by Dr. Pete Lollar, Emory University) and rabbit anti-BiP IgG (Abcam) were diluted in Near IR

Blocking Buffer to final concentrations of 1 and 2.25  $\mu\text{g/ml}$ , respectively. 512G has been shown to have 94% cross-reactivity between human and porcine fVIII by enzyme-linked immunosorbent assay (data not shown). The cells were incubated with the antibodies for 2 h at room temperature with gentle shaking and then washed 4 times for 5 min each with 0.1% Tween 20 (Sigma) in PBS. A secondary antibody staining solution of 10  $\mu\text{g/ml}$  of IRDye 488, 10  $\mu\text{g/ml}$  of IRDye 549 (Jackson ImmunoResearch), and 0.1% Tween 20 were prepared in Near IR Blocking Buffer. The cells were incubated in this solution, protected from light, for 1 h at room temperature and then washed 4 times for 5 min each with 0.1% Tween 20 in PBS. RNase A (Qiagen) then was added to each well at a final concentration of 0.1 mg/ml in PBS, incubated for 10 min at 37 °C, then washed 2 times with PBS. Fifty microliters of 20  $\mu\text{M}$  Draq-5 (Biostatus Ltd.) was added to each well, incubated for 5 min, and then washed twice with PBS. Coverslips were mounted using Prolong Gold anti-fade reagent (Invitrogen) and allowed to cure overnight before imaging on a Nikon Eclipse Ti microscope. Image processing and analysis was performed used NIS Elements AR software from Nikon.

**Protein Stability Assay**—Purified rhfVIII and rpfVIII were generated as previously described (4), and diluted to 0.031 mg/ml in 20 mM HEPES, 5 mM  $\text{CaCl}_2$ , 150 mM NaCl, and 0.01% Tween 80. Two and one-half microliters of 40 $\times$  Sypro Orange Protein Gel Stain (Sigma) was added to 17.5  $\mu\text{l}$  of the diluted protein. Twenty microliters of the protein/stain mixture was loaded into a 96-well optical PCR plate (Thermo), and heated from 27 to 96 °C at a rate of 1 °C every 88 s. The fluorescence was read on an Applied Biosystems 7500 Real-time PCR apparatus using the Rox filter and normalized against a buffer only control.

**Cloning Lentivector BiP**—BiP/GRP78 was cloned into Bluescript KS- using total RNA from 293T cells as previously described (22). The Bluescript KS- BiP/GRP78 and a Lenti rpfVIII plasmid were digested with KpnI and BamHI (New England Biolabs). The BiP/GRP78 and Lenti backbone fragments were gel purified using a QIAquick Gel Extraction Kit (Qiagen) and ligated using T4 DNA ligase (Promega).

**Cloning Lentivector XBP1s-flag**—The lentivector XBP1s-flag plasmid (Addgene) was digested with XmaI, StuI, and SnaBI (New England Biolabs). A lenti-cytomegalovirus backbone was digested with SnaBI. The products were gel purified and the lenti-cytomegalovirus product was treated with shrimp alkaline phosphatase (Promega). The lenti-cytomegalovirus product was then digested with XbpI (New England Biolabs). The XBP1s-flag was then ligated into the lentibackbone using T4 DNA ligase.

**Lentivirus Production**—Lentivirus was produced by cotransfecting 80  $\mu\text{g}$  of BiP, BiP shRNA (Open Biosystems), or XBP1s lentiviral expression plasmid, 40  $\mu\text{g}$  of vesicular stomatitis virus glycoprotein and 40  $\mu\text{g}$  of PAX plasmids into 293T cells grown to 70% confluence in a triple flask (Nunc) using 130  $\mu\text{l}$  of 10 mM PEI MAX (Polysciences, Inc.) in 150 ml of DMEM/F-12 supplemented with 10% FBS. The medium was changed 24 h after transfection, and the supernatant containing the virus was collected every 24 h for 3 days after transfection. The supernatant was subjected to centrifugation overnight at 10,000  $\times g$  at 4 °C.

The viral pellet was resuspended in 4.5 ml of DMEM/F-12, 10% FBS, penicillin/streptomycin, aliquoted, and frozen at  $-80^{\circ}\text{C}$ . The concentrated vector was titered by transducing BHK cells with increasing vector volumes. At 72 h post-transduction, genomic DNA was isolated and DNA copy number was determined by quantitative PCR as described previously (9). Lentiviral particle titers were determined using primers designed specifically to amplify the LTR region of the lentiviral backbone (forward: 5'-ACT TGA AAG CGA AAG GGA AAC-3', reverse 5'-CGC ACC CAT CTC TCT CCT TCT-3').

**Measurement of BiP Transcript Expression**—Relative quantities of BiP transcripts were determined using quantitative real-time PCR. A standard curve was generated using dilutions of the lenti-BiP expression plasmid. PCR were performed using BiP specific primers (forward, 5'-TTG AAT GGC TGG AAA GCC ACC AAG-3' and reverse, 5'-AGG GCC TGC ACT TCC ATA GAG TTT-3') (IDT) as previously described (6).

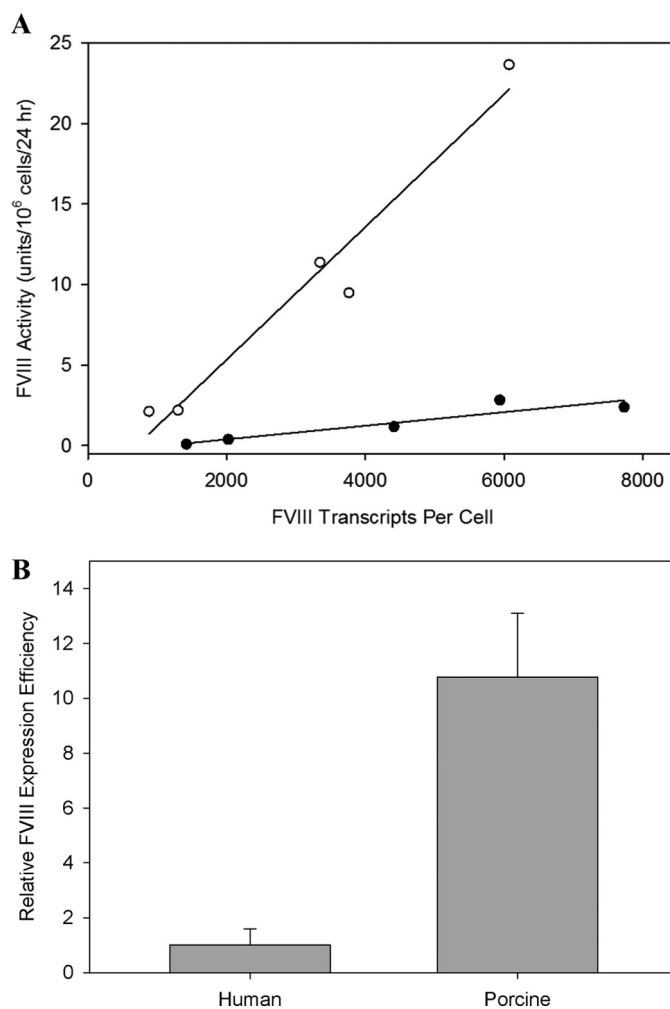
**Measurement of XBP1 Splicing**—One microgram of RNA was reverse transcribed using the Quantitect Reverse Transcriptase Kit (Qiagen) with a 30-min incubation using an XBP1 specific primer (reverse, 5'-TGA CAG GGT CCA ACT TGT CCA GAA-3') to reverse transcribe only the spliced and unspliced isoforms of XBP1 RNA. The reverse transcription product was amplified using either hamster XBP1s-specific primers (forward, 5'-CTG ATG CCG CAG GTG CA-3' and reverse, 5'-TGA CAG GGT CCA ACT TGT CCA GAA-3') or primers that amplified both the spliced and unspliced XBP1 isoforms (forward, 5'-GCT GGA ACA GCA AGT GGT GGA TTT-3' and reverse, 5'-TAC TCC ATT TCC CTT GGA CTC CGT-3') as previously described (6). Dilution curves were prepared using dilutions of reverse transcription product to verify the efficiency of the two sets of primers. Relative quantities of XBP1s *versus* total XBP1 were determined using the  $\Delta C_t$  method.

**Measurement of fVIII Transcript Levels**—fVIII transcript levels were determined using quantitative real-time PCR as previously described (6).

## RESULTS

**RFVIII Expression**—Previously, we reported that rpfVIII is expressed at significantly greater levels than rhfVIII from multiple heterologous expression systems tested as well as from *in vivo* systems employing viral vector nucleic acid transfer strategies (4–12, 24). In the current study, we relied on the use of a BHK expression system that has been described previously and is used commercially (4, 6–8, 25). A panel of rhfVIII and rpfVIII expressing BHK clones were assayed for fVIII activity and fVIII transcript levels (Fig. 1A). Quantitative PCR analysis showed that, despite large differences in fVIII secretion, there was no significant difference in mean fVIII transcript levels observed between rhfVIII and rpfVIII expressing clones ( $p = 0.44$ ). Linear regression analysis demonstrated a significant correlation between fVIII transcripts and fVIII activity for both rhfVIII and rpfVIII ( $p < 0.05$ ). Collectively, these data show a significant differential in fVIII activity per transcript between rhfVIII and rpfVIII expressing clones ( $p < 0.005$ ) (Fig. 1B).

**UPR Induction**—Preliminary analysis of sets of BHK clones expressing various levels of rhfVIII or rpfVIII revealed that

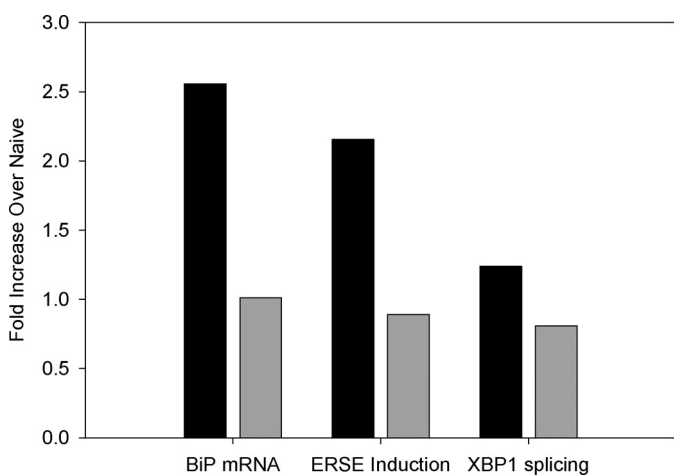


**FIGURE 1. Porcine fVIII is secreted more efficiently than human fVIII.** A, data from individual BHK clones are presented as fVIII activity *versus* fVIII transcript levels per cell. Closed and open circles represent rhfVIII and rpfVIII expressing clones, respectively. B, data from individual clones are presented as fVIII activity per 1000 fVIII transcripts normalized to rhfVIII.

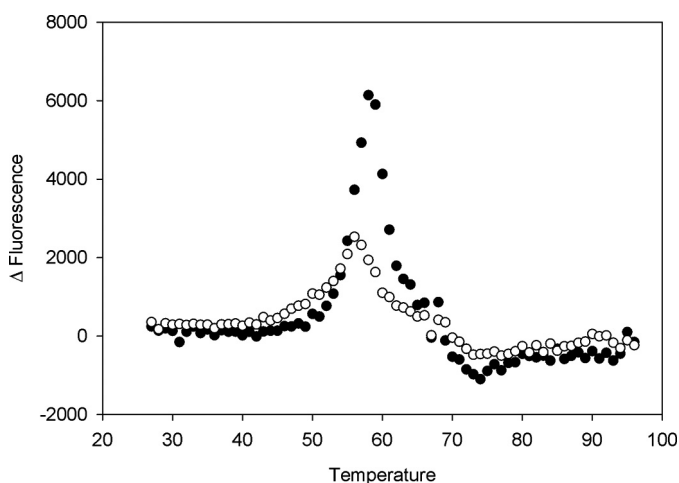
expression of either fVIII molecule led to induction of UPR to an extent proportional to the relative fVIII secretion within the set (data not shown). However, the fVIII secretion levels only overlapped at the lower and higher ends of rpfVIII and rhfVIII secretion, respectively. Therefore for direct comparison, BHK clones expressing similar levels of rhfVIII and rpfVIII ( $\sim 2.5$  units/24 h/10<sup>6</sup> cells) were examined at greater depth for UPR induction (Fig. 2). Interrogation of several established UPR-associated pathways was performed to identify possible differences in the engagement of UPR between the rhfVIII and rpfVIII expressing clones. First, quantitative PCR analysis of BiP transcript levels was performed on each clone. BiP transcript levels were found to be elevated 2.6-fold over control BHKs in the rhfVIII expressing clones, whereas the BiP transcript levels in the rpfVIII expressing clones were comparable with naive BHK cells. Second, transfection of an ERSE inducible reporter revealed 2.2-fold higher luciferase reporter activity in the rhfVIII expressing clones than was observed in non-fVIII expressing, control BHK cells, or the rpfVIII expressing cells. Third, analysis of XBP1 splicing demonstrated a 1.2-fold increase in the rhfVIII expressing clones over naive BHKs,



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**FIGURE 2. Human fVIII is a stronger inducer of UPR than porcine fVIII.** Cells expressing similar levels of rpfVIII and rhfVIII ( $\sim 2.5$  units/24 h/ $10^6$  cells) were assayed for UPR induction. *Black and gray bars* represent rhfVIII- and rpfVIII-expressing cells, respectively. BiP transcript levels relative to naive BHK control cells were determined by one-step quantitative RT-PCR. Induction of ER stress element activity in fVIII expressing cells relative to naive control BHKs was determined by ERSE-inducible luciferase activity measurement. Levels of XBP1 splicing were determined by two-step quantitative RT-PCR.



**FIGURE 3. Human and porcine fVIII display similar thermal denaturation.** Protein unfolding was monitored by a reporter that fluoresces when exposed to hydrophobic substrates. The reporter was added to purified rhfVIII and rpfVIII and then heated. *Closed and open circles* represent rhfVIII- and rpfVIII-expressing cells, respectively.

whereas the rpfVIII expressing clones showed a small decrease in XBP1 splicing.

**Thermal Stability of RhfVIII and RpfVIII**—To address whether the increased fVIII production seen from the rpfVIII-expressing clones over the rhfVIII-expressing clones could be due to a differential stability of the proteins, Sypro Orange, a reporter that fluoresces when exposed to hydrophobic substrates, was added to purified samples of rhfVIII and rpfVIII. Protein unfolding was monitored by heating the samples from 27 to 96 °C and the rate of change of fluorescent signal with respect to temperature was monitored. RpfVIII and rhfVIII displayed similar  $T_m$  of 56 and 58 °C, respectively (Fig. 3). However, it was noted that that rhfVIII showed greater baseline fluorescence and greater  $\Delta$ fluorescence than rpfVIII.

**Immunofluorescence Microscopy of FVIII Biosynthesis**—Analysis of the rhfVIII and rpfVIII expressing clones using

immunofluorescence microscopy revealed differences in the intracellular distribution of fVIII. By immunolabeling BiP (ER) and fVIII (Fig. 4, A and B), it was observed that rhfVIII and rpfVIII both co-localize with BiP within the ER. However, an abundance of rpfVIII also was present surrounding the nucleus in the location typically corresponding to the Golgi apparatus, which was not observed in the rhfVIII expressing clone. To confirm localization of rpfVIII within the Golgi, Golgi was detected using fluorescently labeled wheat germ agglutinin (Fig. 4, C and D, red). Upon Golgi labeling, a yellow area representing co-localization of fVIII and Golgi became apparent in the rpfVIII expressing clone but was absent in the rhfVIII expressing clone. An apparent difference was also seen in the morphology of the Golgi between the rhfVIII and rpfVIII expressing clones. The Golgi in the rpfVIII expressing clone showed tight perinuclear staining, whereas the Golgi in the rhfVIII expressing clone was more rounded and extended into the ER space. The Golgi of control BHKs appeared morphologically more similar to that of the rpfVIII expressing clone.

**Overexpression of BiP**—Previously, others demonstrated an interaction between rhfVIII and BiP (26, 27). To determine whether BiP could be affecting the differential expression of rhfVIII and rpfVIII, we constructed a lentivector-encoding BiP and transduced the fVIII-expressing clones. Post-transduction, a differential response was observed between the rhfVIII and rpfVIII expressing clones. A m.o.i.-dependent increase in BiP transcripts was seen in both sets of clones and at the highest m.o.i., 7.5- and 4.1-fold increases in BiP transcripts were observed for the rhfVIII and rpfVIII expressing clones, respectively (Fig. 5A). In the rhfVIII-expressing clones, a m.o.i.-dependent decrease in activity to 58% of the original level was observed. The rpfVIII-expressing clones showed no significant change in fVIII production upon BiP overexpression (Fig. 5B). Quantitative PCR revealed no change in fVIII transcript levels following BiP overexpression (Fig. 5C).

**Expression of BiP shRNA**—To further validate the results observed upon BiP overexpression, we constructed a lentivector-encoding BiP shRNA and transduced control (non-fVIII expressing), rhfVIII- and rpfVIII-expressing clones. A differential, but still m.o.i.-dependent decrease in BiP transcripts was seen in both sets of clones. At the highest m.o.i. tested, 69 and 53% decreases in BiP transcripts were seen in the rhfVIII- and rpfVIII-expressing clones, respectively (Fig. 6A). In the rhfVIII-expressing clones, a m.o.i.-dependent increase in fVIII activity up to 2-fold at m.o.i. = 4 was observed. The rpfVIII-expressing clones showed a similar, but less dramatic increase up to 1.3-fold at m.o.i. = 4 (Fig. 6B). Furthermore, quantitative PCR revealed a m.o.i.-dependent decrease in fVIII transcripts upon BiP knockdown in both the rhfVIII- and rpfVIII-expressing clones with a maximal decrease of  $\sim 65\%$  (Fig. 6C).

**Overexpression of XBPs**—To investigate whether XBPs plays a beneficial or inhibitory role in fVIII biosynthesis, we constructed a lentivector encoding constitutively expressed XBPs and transduced the fVIII-expressing clones. A similar response was seen among the rhfVIII- and rpfVIII-expressing clones following transduction. No change was observed in either fVIII or BiP transcripts in either clone post-transduction (data not shown). However, following transduction at the high-

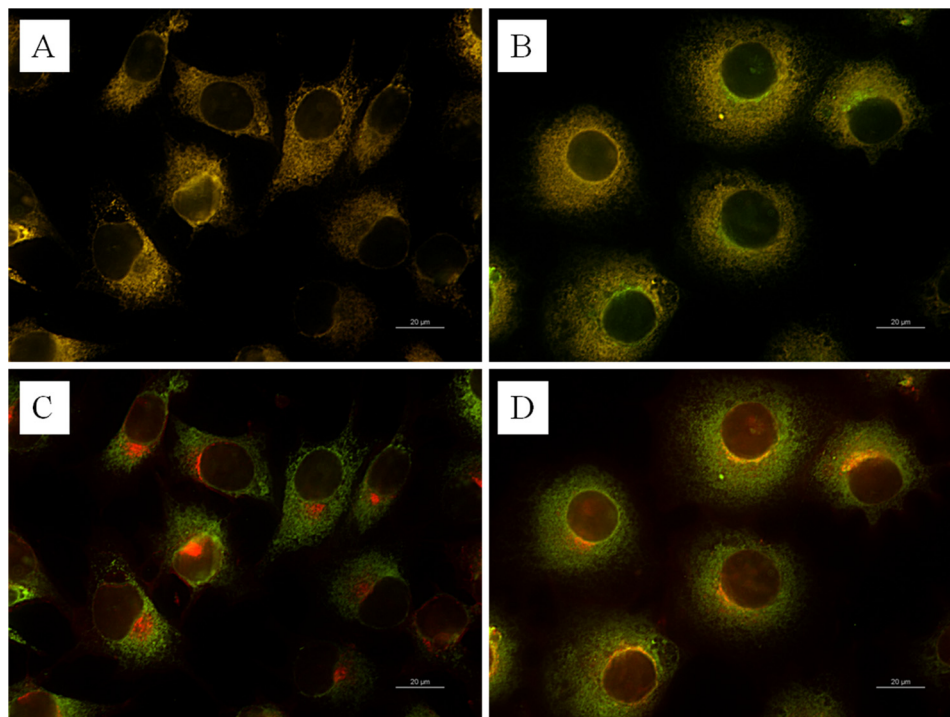


FIGURE 4. **Porcine fVIII is more prevalent in the Golgi than human fVIII.** Immunofluorescence cytochemistry microscopy was performed on rh (A and C) and rp (B and D) fVIII expressing BHK cells. Reactive antibodies targeting BiP (red in A and B), fVIII (green in A–D), and Golgi (red in C and D) were utilized. Co-localization of fVIII and BiP (A and B) or Golgi (C and D) is represented by the yellow color.

est m.o.i., both sets of clones showed a 50% increase in fVIII secretion (Fig. 7).

## DISCUSSION

Porcine fVIII (rpfVIII) is expressed at levels 10–100-fold higher than human fVIII both from *in vitro* heterologous expression systems and *in vivo* using gene transfer methods (4–12, 24). In the current study, we demonstrate that, per transcript, rpfVIII ultimately is secreted 11-fold more efficiently than rhfVIII. Previous studies dismissed increased transcriptional rates, enhanced nuclear export, or improved mRNA stability as likely mechanisms for the higher levels of biosynthesis seen by rpfVIII over rhfVIII (5). Here, we demonstrate that rhfVIII and rpfVIII differentially engage the UPR pathway on a per secreted molecule basis, with rhfVIII expression producing induction for all UPR markers that were probed and rpfVIII showing significantly lesser or undetectable engagement at the levels of fVIII expression investigated (Fig. 2). These findings suggest that, despite 83% sequence identity, rhfVIII and rpfVIII display biosynthetically significant differences in folding kinetics, conformation, or stability that likely affect the interaction with BiP as well as possibly other UPR components.

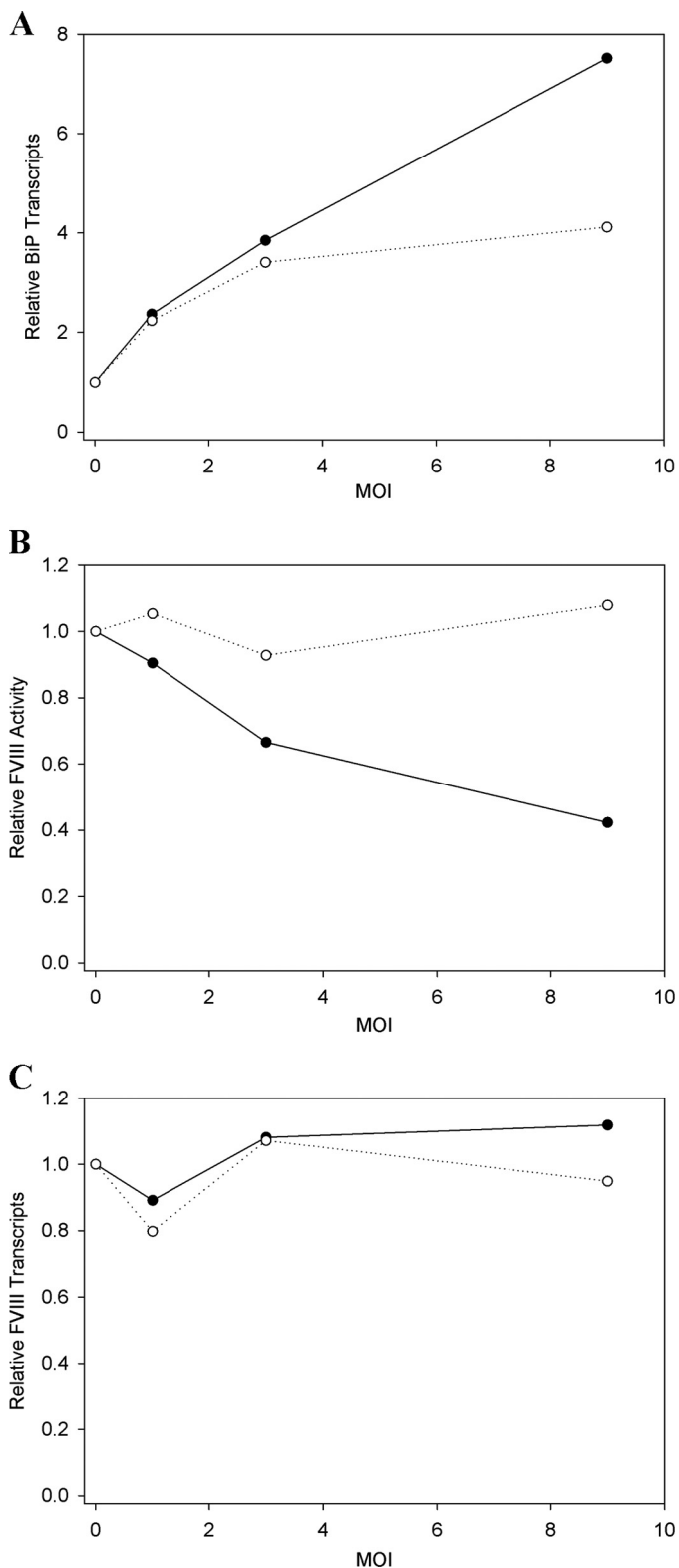
Using immunofluorescence imaging, differences in the intracellular localization of rhfVIII and rpfVIII were uncovered (Fig. 4). The high degree of colocalization of rpfVIII with the Golgi marker, wheat germ agglutinin, was not seen for rhfVIII, suggesting that rhfVIII is not trafficked efficiently from the ER to the Golgi. This finding can be interpreted one of two ways. One interpretation is that rhfVIII may be inefficiently trafficked to the Golgi, leading to an accumulation of rhfVIII in the ER, thus causing induction of UPR due to significant protein accumula-

tion. A second interpretation is that, upon co-translational insertion into the ER lumen, 1) rhfVIII rapidly engages BiP or other UPR partners; 2) is held up in the ER by quality control mechanisms such as calnexin and calreticulin; and 3) eventually is subjected to ER associated degradation as opposed to properly folding and trafficking into the Golgi.

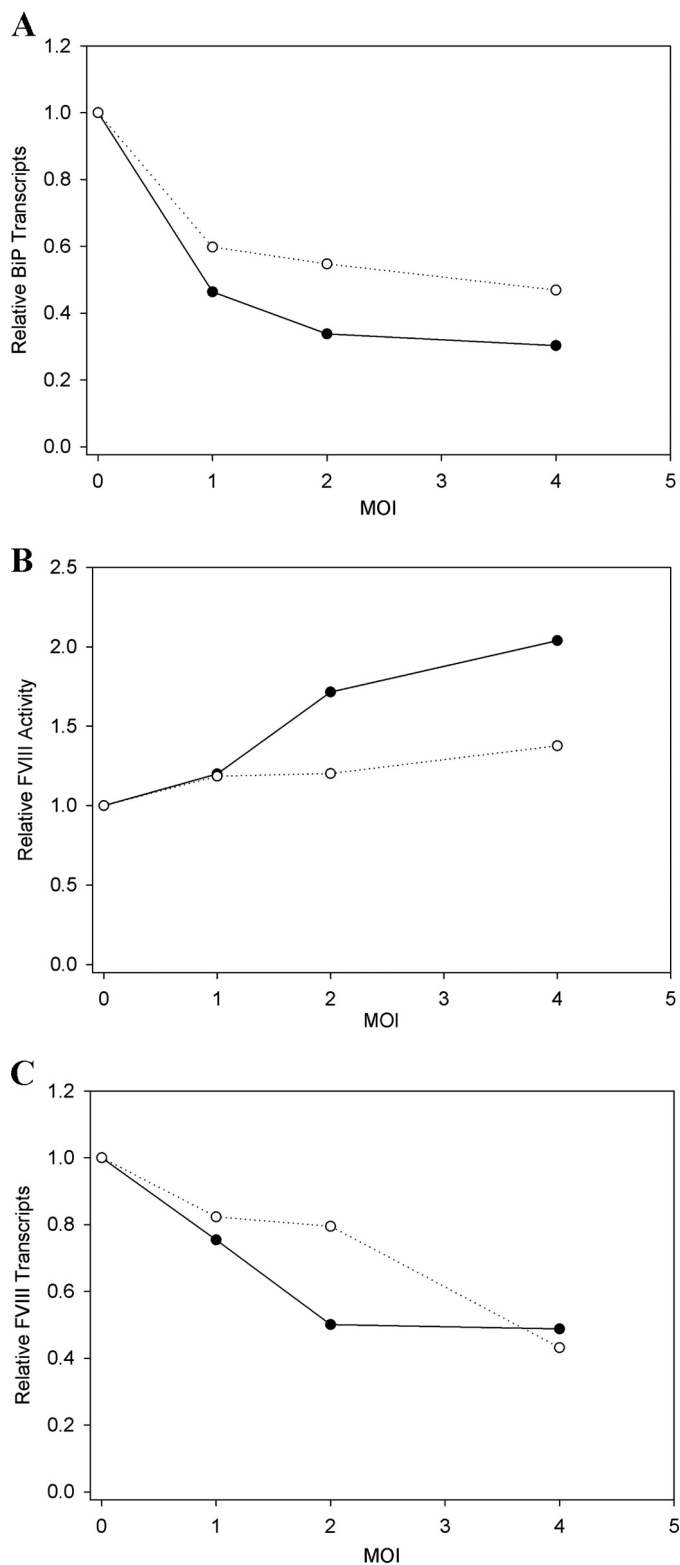
Exogenous modulation of UPR activity using lentivectors uncovered additional differentials between rhfVIII and rpfVIII biosynthesis. Constitutive BiP overexpression was found to decrease expression of rhfVIII, whereas having no effect on rpfVIII expression (Fig. 5). In contrast, knockdown of BiP levels using shRNA was found to enhance expression of rpfVIII only slightly, whereas nearly doubling the expression of rhfVIII. Of note, this finding occurred despite a decrease in overall fVIII transcript levels. It is known that BiP is an essential protein, at least in mice, as its deletion results in peri-implantation lethality (17). Therefore, it is possible that the decreases observed for both human and porcine fVIII mRNA levels upon BiP knockdown are the result of general cellular toxicity. Taken together, these data suggest that rpfVIII biosynthesis is relatively insensitive to BiP concentration, whereas rhfVIII biosynthesis displays a high degree of BiP concentration dependence. Given the increase seen in both rhfVIII and rpfVIII expressing cells, it appears that BiP does interact with both rhfVIII and rpfVIII and this interaction may be responsible, at least partially, for the low overall expression of both species' fVIII.

It also is possible that UPR could be beneficial to fVIII biosynthesis by expanding the capacity of the ER through chaperone up-regulation. Therefore, UPR was induced in BHKs expressing recombinant fVIII using the potent UPR inducer,

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**FIGURE 5. Overexpression of BiP inhibits human fVIII secretion but not porcine.** Closed and open circles represent rhfVIII- and rpfVIII-expressing cells, respectively. *A*, fold-changes in BiP transcripts were determined by quantitative RT-PCR. *B*, FVIII activity in the conditioned medium was determined by one-stage coagulation assay. *C*, relative levels of fVIII transcripts were determined by quantitative RT-PCR. All values were normalized to the respective untransduced control BHK cells expressing either rhfVIII or rpfVIII.



**FIGURE 6. BiP shRNA expression enhances human fVIII secretion more than porcine fVIII.** Closed and open circles represent rhfVIII and rpfVIII expressing cells, respectively. *A*, relative levels of BiP transcripts were determined by quantitative RT-PCR. *B*, fold-changes in fVIII activity were determined by a one-stage coagulation assay. *C*, relative levels of fVIII transcripts were determined by quantitative RT-PCR.

thapsigargin, and a dose responsive decrease in fVIII expression was observed (data not shown). In XBP1s has been shown to inhibit UPR induced translational arrest and cause up-regula-

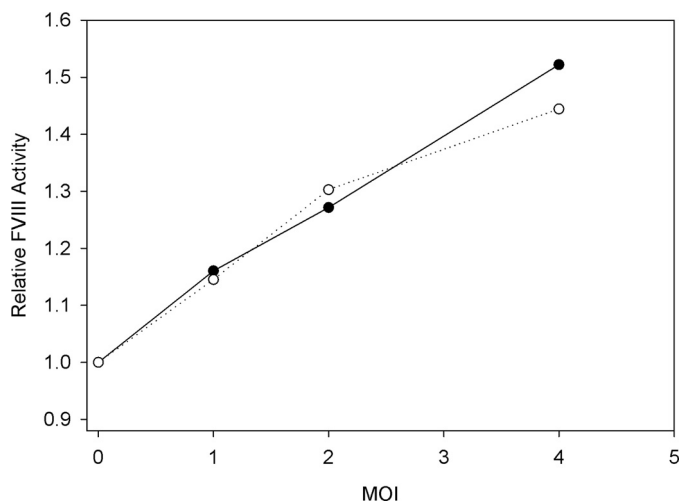


FIGURE 7. XBP1s enhances human and porcine fVIII secretion. Closed and open circles represent rhfVIII- and rpfVIII-expressing cells, respectively. Fold-changes in fVIII activity were determined by a one-stage coagulation assay of the conditioned media.

tion of glycosylation and disulfide bond formation (19). Constitutive expression of XBP1s was found to be beneficial for the expression of both rhfVIII and rpfVIII (Fig. 7). Although the effects of XBP1s may not explain the differential expression of rhfVIII and rpfVIII, it does pose a putative target for the improvement of commercial recombinant fVIII manufacturing.

The current studies demonstrate proof of principle that UPR intervention can be used to improve heterologous expression of fVIII, especially rhfVIII. Further studies are needed to investigate if the coexpression of BiP shRNA and XBP1s expression are additive, synergistic, or antagonistic. Here, we only looked at two of the numerous proteins involved in UPR. Further investigation may uncover more aspects of UPR that can be exploited to provide higher levels of expression. The low level expression of rhfVIII in mammalian expression systems is associated with the high cost of treatment for hemophilia A (23). An increase in expression through better understanding of fVIII biosynthesis could substantially reduce the cost of treatment for persons with hemophilia A.

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