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ORIGINAL ARTICLE

Functional factor VIII made with von Willebrand factor at high levels in transgenic milk

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Summary. *Background:* Current manufacturing methods for recombinant human factor VIII (rFVIII) within mammalian cell cultures are inefficient, hampering the production of sufficient amounts for affordable, worldwide treatment of hemophilia A. However, rFVIII has been expressed at very high levels by the transgenic mammary glands of mice, rabbits, sheep, and pigs. Unfortunately, it is secreted into milk with low specific activity, owing in part to the labile, heterodimeric structure that results from furin processing of its B domain. *Objectives:* To express biologically active rFVIII in the milk of transgenic mice through targeted bioengineering. *Methods:* Transgenic mice were made with a mammary-specific FVIII gene (*226/N6*) bioengineered for efficient expression and stability, encoding a protein containing a B domain with no furin cleavage sites. 226/N6 was expressed with and without von Willebrand factor (VWF). 226/N6 was evaluated by ELISA, SDS-PAGE, western blot, and one-stage and two-stage clotting assays. The hemostatic activity of immunoaffinity-enriched 226/N6 was studied *in vivo* by infusion into hemophilia A knockout mice. *Results and conclusions:* With or without coexpression of VWF, 226/N6 was secreted into milk as a biologically active single-chain molecule that retained high specific activity, similar to therapeutic-grade FVIII. 226/N6 had $>$ 450-fold higher IU mL^{-1} than previously reported in cell culture for rFVIII. 226/N6 exhibited similar binding to plasma-derived VWF as therapeutic-grade rFVIII, and intravenous infusion of transgenic 226/N6 corrected the bleeding phenotype of hemophilia A mice. This provides proof-of-principle for the study of expression of 226/N6 and perhaps other single-chain bioengineered rFVIIIs in the milk of transgenic livestock.

Keywords: factor VIII, hemophilia, mammary gland, modified B domain, recombinant protein, transgenic animal, von Willebrand factor.

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Introduction

Hemophilia A is an X-linked, inherited disorder of blood coagulation, primarily caused by deficiency or dysfunction of factor VIII, that affects approximately one in every 5000–10 000 males [1–3]. FVIII is present at only 100–200 ng mL^{-1} in blood plasma, and is a post-translationally complex, large glycoprotein of about 260–280 kDa [4–6]. FVIII acts as an essential cofactor for FIX in the coagulation cascade to amplify the activation of FX $>$ 1000-fold. The structural complexity of FVIII [7] remains at the core of therapeutic problems such as abundance and the frequently encountered inhibitory immune responses to intravenous therapy. Thus, both the development of universal prophylactic and non-intravenous, low-bioavailability treatments in economically developed countries and routine access in developing countries are precluded [1,8,9].

The biosynthesis and secretion of FVIII into blood plasma by hepatocytes and recombinant FVIII (rFVIII) by mammalian cells are greatly and similarly inefficient, owing to limitations in transcription [10–12], post-translational processing, and translocation from the endoplasmic reticulum to the Golgi apparatus [13,14]. The most biosynthetically restricted rFVIII structure is the analog of native FVIII containing its full-length (907 amino acids, 90 kDa) B domain (FL-rFVIII). The B domain is processed by furin at two different sites, leading to a complex mixture of labile [15], metal ion-stabilized heterodimers (Fig. S1). Thus, all therapeutic-grade plasma-derived FVIIIs (FL-FVIIIs) and FL-rFVIIIs are heterodimeric and contain no single-chain species [7]. von Willebrand factor (VWF) helps to stabilize the labile, heterodimeric structure of both FL-FVIII in the blood circulation and of FL-rFVIII in cell culture media. Although VWF does not increase the expression levels of FL-rFVIII, the presence of VWF in cell culture media increases the accumulation of FVIII activity to about 1–2 IU mL^{-1}, which is about the same as in normal human plasma [16].

We and others have previously reported high concentrations of FL-rFVIII in the milk of transgenic mice [17], rabbits [18,19], sheep [20], and pigs [21], showing that the mammary gland can efficiently secrete rFVIII with a full-length B domain. However, the specific activity of the FL-rFVIII was very low, at about 0–10% of expected levels (Table S1). The native

appearance of the FL-rFVIII heavy and light chains found in these milks suggested that the conformation of the assembled heterodimer, and not necessarily proteolytic degradation, was the main cause of the low activity in milk (Fig. S2) [21].

Unlike previous studies, in which FL-rFVIII was expressed in milk, in this study we expressed a bioengineered rFVIII with a modified B domain (226/N6) having no furin sites in the presence and absence of the potentially stabilizing influence of recombinant VWF (rVWF) coexpressed in mouse milk. The consensus sites for furin cleavage at Arg1313 and Arg1648 were removed by truncating the B domain to the first 226 amino acids (Fig. S1B). We provide the first report of rFVIII made at high levels in transgenic milk with native procoagulant activity.

Materials and methods

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Michigan and Virginia Polytechnic Institute and State University.

DNA constructs

The *226/N6* (*WAP7FVIII-226/N6*) construct was assembled by altering the *Kpn*I site of pUCWAP6 [22] by introducing a *Sal*I site immediately downstream of the 4.1-kbp whey acidic protein (WAP) promoter and ahead of the 1.7 kbp of mouse WAP 3′-untranslated region (UTR) containing the coding sequence for the polyadenylation signal to produce pUC-WAP7. The sequence for *FVIII-226/N6* was removed by restriction enzyme digestion with *Sal*I and *Xho*I from pMT_2 226/N6, as described previously [14], and introduced into the *Sal*I site of pUCWAP7, yielding the pUCWAP7FVIII-226/N6 plasmid. The plasmid pUCWAP6VWF containing the *WAP6VWF* expression construct (gift from H. Lubon, American Red Cross, Rockville, MD, USA) contained the cDNA for VWF placed between the 4.1-kbp WAP promoter and the 1.7-kbp 3′-UTR. The plasmid designated PPL456, containing the β-lactoglobulin (BLG)-driven α_1-antitrypsin (AAT) construct, which has been previously described [23], was a gift from D. Ayares (Revivicor, Blacksburg, VA, USA). The *WAP7F-VIII-226/N6* was released by *Not*I. The *WAP6VWF* was released by digestion with *Not*I and *Sfi*I. The *BLG-AAT* was released by digestion with *Not*I and *Sal*I. All constructs were purified by extraction prior to microinjection with a Nucleospin Extract kit (Clonetech Laboratories, Palo Alto, CA, USA).

Transgenic mouse generation, identification, and milk collection

Transgenic mice were generated by pronuclear microinjection of purified DNA constructs, as previously described [24], at the Transgenic Core facility at the University of Michigan. Southern blot analysis was performed as previously described [21]. PCR probes (KPL, Gaithersburg, MD, USA) are shown in Fig. S3A. The primers for 500 base pair PCR products were: for WAP, WAPprobeS1 (gcatgctcacactcaacagg)/WAPprobe-A1 (taagagtgtggaggcgcttg); and for AAT, the same primers as for PCR, BLG-S1/AAT-A1. Milk was collected and defatted as previously described [24]. Samples were stored at − 70 °C.

FVIII biochemical analysis

FVIII activity was measured with a one-stage activated partial thromboplastin time (APTT) clotting assay on an MLA Electra 750 fibrinometer by reconstituting human FVIII-deficient plasma (George King Bio-Medical, Overland Park, KS, USA), and a modified two-stage assay utilizing the COAMATIC Factor VIII (Chromogenix, Milan, Italy), according to the manufacturer's instructions. FVIII antigen levels were determined with an ELISA kit (Affinity Biologicals, Ancaster, ON, Canada), on the assumption of an FVIII concentration of 200 ng mL^{-1} per 1 IU mL^{-1} of activity (5000 IU mg^{-1}); 1 IU mL^{-1} is the defined concentration of FVIII activity in normal human plasma, which contains an average concentration of 100–200 ng mL^{-1}.

Immunoaffinity chromatography (IAC) enrichment of 226/N6

Defatted milk was diluted and incubated overnight with anti-heavy chain FVIII monoclonal antibody-conjugated sepharose beads (kindly provided by Baxter BioScience, Deerfield, IL, USA). The enrichment procedure was carried according to the protocol described previously [25], with and without wash steps with 250 mM $CaCl_2$ and $MgCl_2$ to disrupt FVIII:VWF complexes [26] prior to elution of FVIII. One milligram per milliliter of AAT was added as a stabilizer after IAC enrichment. 226/N6 from CHO cells was enriched from stably transfected CHO cell lines as described previously [14].

Immunoblotting

Non-reduced and reduced samples were evaluated by SDS-PAGE on 4–12% NuPage Bis-Tris gels (Invitrogen Carlsbad, CA, USA), and then electroblotted onto poly(vinylidene fluoride) membranes (Millipore, Billerica, MA, USA). Blots were probed with polyclonal antibodies against FVIII (F0016-10; US Biological, Swampscott, MA, USA), VWF (V2700-04B; US Biological), or AAT (A2298-27H; US Biological).

FVIII–VWF binding ELISA

226/N6 and rFVIII (Advate; gift from Baxter) were incubated with 1% FVIII-deficient human plasma (George King Bio-Medical). Triplicate samples were incubated with an anti-FVIII antibody (BO2C11; gift from M. Jacquemin, University of Leuven, Belgium) that inhibits VWF binding [27] prior to incubation with 1% FVIII-deficient human plasma. Samples were loaded into 96-well plates precoated with anti-FVIII antibody (F8C-EIA-C; Affinity Biologicals). Anti-VWF antibody (p0266; Dako, Carpenteria, CA, USA) was used to detect VWF binding. Absorbance was read at 490 nm.

In vivo tail clip assay in hemophilia A (HA) mice

HA exon 16 knockout mice [28] were subjected to a tail clip bleeding assay. Mice at least 10 weeks of age were injected with IAC-enriched FVIII proteins. C57BL/6 and HA mouse littermates were used as controls for injection with lactated Ringer's (LR) solution. Mice were injected with 80 IU kg^{-1} body weight of FVIII in 100 μL LR solution via the tail vein. Ketamine 85 mg kg^{-1} with xylazine 5 mg kg^{-1} was injected intraperitoneally to induce anesthesia. After 5 min, tails were cut at 1.5-mm diameter from tail tips, and then immediately submerged in 14-mL conical tubes filled with saline at 37 °C. Blood from the tail was collected at 10 min. Blood loss was quantified by the difference in tube weight between time 0 and 10 min. Mice were then killed.

Statistical analysis

Data are presented as mean ± standard deviation, except for bleeding data, which are presented as median ± standard deviation. A single-factor analysis of variance (ANOVA) was performed to compare blood loss results. Student's *t*-tests (two-tailed), assuming unequal variances, were used to compare each treatment group with control mice. An alpha level of 0.05 was used for all statistical analysis.

Results

Production and selection of transgenic mice by expression level

We made monogenic and bigenic *226/N6* mice with three different transgenes (Fig. S3) to help test the following hypotheses: (i) single-chain rFVIII would be expressed at a range of concentrations in milk similar to or higher than that of FL-rFVIII; (ii) coexpressed rVWF would complex 226/N6 and help stabilize single-chain rFVIII made in milk, as it does in plasma and cell culture; and (iii) single-chain rFVIII would have higher specific activity than FL-rFVIII when expressed in milk. An optimized *BLG-AAT* gene was used to make lineages of bigenic and trigenic *226/N6* mice in an effort to mitigate transcriptional silencing, which occurs in > 60% of cases of mammary expression in transgenic mice. Silencing was decreased to about 25% in mice coinjected with the *BLG-AAT* transgene [29,30]. Twenty different potential founder animal litters were made by outbreeding with control animals. Transgenic mice maintained a natural lactation length of about 17 days, and had normal litter growth. About 50–100 μL of milk was obtained per milking.

Detectable levels of rFVIII and activity were present in some mice monogenic for *226/N6* alone, whereas no rFVIII protein or activity was detected in milk from mice bigenic for *226/N6* and *rVWF* (Table 1). In the presence of the *BLG-AAT* milk gene, both the level and activity of *226/N6* were much higher for bigenic (*226/N6* and *BLG-AAT*) and trigenic (*226/N6*, *rVWF*, and *BLG-AAT*) lineages than for monogenic *226/N6* mice. For these bigenic and trigenic mice containing the *BLG-AAT* gene, AAT was expressed at > 1 mg mL^{-1} (Fig. S4). In summary, whereas transgene silencing probably occurred in some lineages, the concentration range of 226/N6 found in the milk of all mice was similar to previously reported concentration ranges for FL-rFVIII made in the milk of mice, rabbits, sheep, and pigs, with the highest concentration of 226/N6 being about 0.2–0.3 mg mL^{-1}.

Table 1 The specific activity of 226/N6 in milk

Mouse genotype, mouse ID	226/N6 concentration (μg mL^{-1})	APTT activity level (IU mL^{-1})	Specific activity (IU mg)
Monogenic mice: *226/N6*			
1960-1702	0.144	0.6	4.167
1926-1742	0.093	0.1	1.075
46-1247	0.066	0.4	6.061
Bigenic mice: *226/N6-rVWF*			
376-8	Not detected	Not detected	
376-12	Not detected	Not detected	
383-49	Not detected	Not detected	
Bigenic mice: *226/N6-BLG-AAT*			
875-234	34	25	735
875-233	6.2	44	7.097
516-46	7.3	13	1.781
406-128-10	83	122	1.470
Trigenic mice: *226/N6-rVWF-BLG-AAT*			
415-101-2	183	678	3.705
415-101-1–2	122	555	4.549

AAT, α_1-antitrypsin; APTT, activated partial thromboplastin time; BLG, β-lactoglobulin; rVWF, recombinant von Willebrand factor. 1 IU mL^{-1} is the defined concentration of FVIII activity in normal human plasma. An average concentration of 200 ng mL^{-1} per mL of normal human plasma is assumed, yielding a theoretical specific activity of 5000 IU mg^{-1}.

Specific clotting activity and analysis by western blot

We studied the transgenic mice with the highest 226/N6 protein expression levels and expanded those lineages. The specific activity was determined directly in the whole milk pools by measurement of FVIII antigen by ELISA and coagulation activity by APTT assay (Table 1). Notably, the 226/N6 specific activities found in the milk from monogenic, bigenic and trigenic mice were all similar and typical of plasma-derived FVIII or FL-rFVIII. Importantly, 226/N6 was also functional without the presence of AAT or rVWF. Because there are few high molecular mass proteins in the milk of mice near or at the molecular mass of 226/N6 or VWF under reducing conditions, quantitative western blotting was possible. The high 226/N6 levels determined by ELISA were confirmed by quantitative western blot analysis under reducing conditions (Fig. S5).

Interactions of 226/N6 with rVWF and VWF

Strong interactions between VWF and FVIII result from conformations stabilized by intramolecular disulfide bonding within FVIII and VWF subunits. The interactions between FL-FVIII and VWF in therapeutic-grade preparations are not appreciably destabilized by treatment with SDS under non-reducing conditions, as shown by the decreased mobility seen in western blot analysis (Fig. 1). Reducing conditions destabilize these binding interactions, resulting in greater mobility that is commensurate with the molecular size of FVIII and VWF monomers, respectively. In contrast, purified preparations of therapeutic-grade FVIII containing no VWF do not show the greatly decreased electrophoretic mobility in SDS-PAGE under non-reducing or reducing conditions. This intramolecular disulfide bridge-dependent phenomenon occurring within the VWF and FVIII molecules was used to help determine the extent of 226/N6 complexation with rVWF in milk. The electrophoretic migration of heterodimers under reducing and non-reducing conditions for therapeutic-grade FL-rFVIII made in CHO cells not containing VWF was similar. The molecular mass of the heterodimers was consistent with the published range of 100–220 kDa (Fig. 1A,B), although they possessed no signal for VWF (Fig. 1C,D).

A trigenic (226/N6, rVWF, and BLG-AAT) mouse milk pool containing 300 μg mL^{-1} of both 226/N6 and rVWF showed a single predominant FVIII signal at about 190 kDa under reducing conditions. The VWF signal had a similar molecular mass (about 320 kDa) as the plasma-derived FVIII:VWF reference under reducing conditions. Importantly, the same shift to a retarded migration of molecular mass > 420 kDa occurred for the trigenic sample under non-reducing conditions as occurred with the plasma-derived FVIII:VWF complex. Bigenic mouse milk containing no rVWF migrated similarly to the trigenic sample under reducing conditions, but did not exhibit the greatly retarded migration under non-reducing conditions. These results show that 226/N6 was secreted as a single chain into milk with or without VWF.

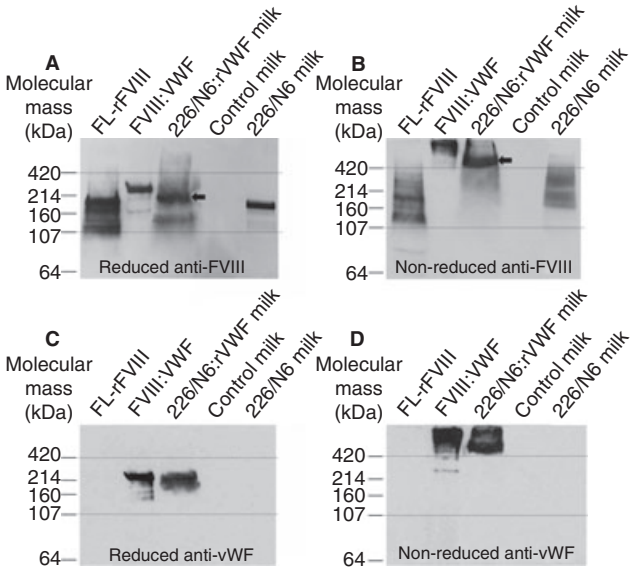

Fig. 1. SDS-PAGE of FL-rFVIII and milk-borne 226/N6 in the presence and absence of von Willebrand factor (VWF) under reducing and non-reducing conditions. Assessment of VWF binding by (A) reduced anti-FVIII, (B) non-reduced anti-FVIII, (C) reduced anti-VWF, and (D) non-reduced anti-VWF SDS-PAGE western blots. All blots contain a molecular mass marker. FL-rFVIII, recombinant FVIII; FVIII:VWF, plasma-derived FVIII:VWF concentrate; 226/N6:rVWF milk, diluted trigenic mouse milk equivalent to 0.07 μL of undiluted milk; Control milk, diluted non-transgenic mouse milk equivalent to 0.07 μL of undiluted milk; 226/N6 milk, diluted bigenic mouse milk equivalent to 0.07 μL of undiluted milk. Arrows indicate primary single-chain 226/N6 species.

IAC was applied to a whey phase made from centrifugally clarified and diluted trigenic mouse milk to provide an infusable preparation enriched in 226/N6 (Fig. 2). The IAC captures FVIII and FVIII:VWF complexes. Trigenic milk was subjected to IAC with loading and wash conditions under which VWF:FVIII complexes were unstable, allowing most of the VWF to be efficiently released before and during immunosorption of 226/N6. Whereas the starting milk contained a 1 : 1 ratio of rVWF to 226/N6, IAC enrichment reduced the stoichiometric ratio of rVWF to 226/N6 in the FVIII eluate to about 0.1. The yield of 226/N6 from trigenic milk containing high levels of 226/N6 was 10%, with an enrichment of 70-fold, and a purity of 39% before addition of AAT for storage. This IAC preparation was similar to an IAC-enriched therapeutic-grade FL-rFVIII made from Recombinate (Table 2). Importantly, the specific activity of the IAC-enriched 226/N6 from trigenic milk was similar to that of the 226/N6 from the original milk and also the FL-FVIII and FL-rFVIII references (Table S2).

IAC was also applied to milk from low-expressing animals that did not coexpress VWF. Although the purity was < 1% (Fig. S6), an enrichment of 16-fold was achieved for 226/N6 at a similar specific activity to that found in the original milk (Table 2). The yield of 226/N6 captured from milk containing 226/N6:VWF complexes was lower than the yields from non-VWF-expressing milk.

Using ELISA, we studied the complexation of human plasma-derived VWF with 226/N6 that could occur in FVIII-deficient plasma derived from a severe HA donor. We used this model to determine whether 226/N6:VWF complexes would form in a context more pertinent to future HA treatment. The IAC-enriched 226/N6 preparations and therapeutic-grade FL-rFVIII were immunocaptured on a microtiter plate. The immobilized anti-FVIII monoclonal antibody has been shown not to interfere with VWF binding. The presence of VWF bound to the immunocaptured 226/N6 was detected with an anti-VWF polyclonal antibody. To determine the specificity of the interaction between 226/N6 and VWF in blood plasma, we preincubated each immunocaptured rFVIII species with and without a monoclonal antibody (B02C11) that binds to the light chain of FVIII, inhibiting FVIII:VWF complexation [27]. In all cases, B02C11 inhibited the VWF signal arising from incubation in plasma to low background levels. Without pretreatment with B02C11, both immunocaptured FL-rFVIII

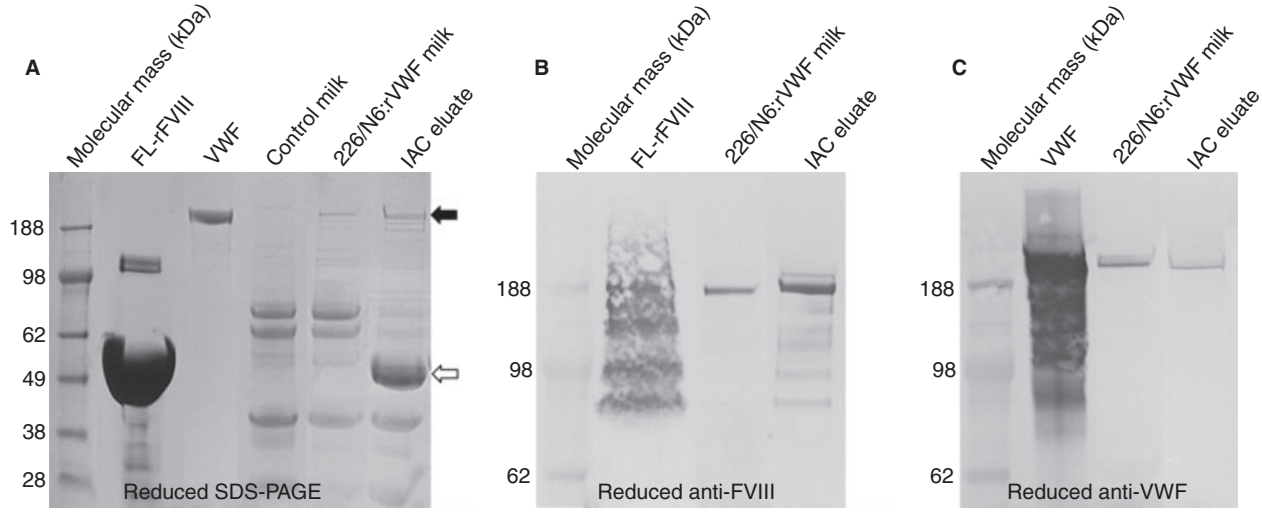

Fig. 2. SDS-PAGE of immunoaffinity chromatography (IAC)-enriched 226/N6. 226/N6 and recombinant von Willebrand factor (rVWF) were IAC-enriched from transgenic mouse milk and separated during the enrichment process. Enrichment of the 226/N6 and rVWF was performed on clarified, diluted milk pooled from six F_3 trigenic mice from the 415-101-2 line, and analyzed by reducing SDS-PAGE (A) and anti-FVIII (B) and anti-VWF (C) western blots. The SDS-PAGE gel (A) contains a molecular mass marker, an FVIII reference (FL-rFVIII; recombinate), a VWF reference (VWF), diluted non-transgenic mouse milk equivalent to 0.07 μL of undiluted milk (Control milk), diluted trigenic mouse milk equivalent to 0.07 μL of undiluted milk (226/N6:rVWF milk), and trigenic milk product enriched by IAC (IAC eluate). The enriched 226/N6 is shown by the closed arrow (⬅). The open arrow (⇦) points to the α_1-antitrypsin (AAT) that was added at 1 mg mL^{-1} to the IAC eluate for storage formulation. Each blot (B and C) contains a molecular mass marker, a reference (FL-rFVIII and/or VWF), trigenic transgenic mouse milk (226/N6:rVWF milk), and IAC-enriched trigenic milk product (IAC eluate).

Table 2 The specific activity of immunoaffinity chromatography (IAC)-enriched 226/N6 from clarified, diluted milk

Sample	Total protein (mg mL^{-1})	FVIII antigen (μg mL^{-1})*	FVIII activity (IU mL^{-1})†	One-stage activity level (IU mg^{-1})	% Purity	% Antigen yield	IAC enrichment
Therapeutic-grade FL-rFVIII (Recombinate)	21.95 ± 3.57	26.35 ± 2.64	90.09 ± 9.22	3.419 ± 634	< 1	NA	NA
IAC-enriched FL-rFVIII (Recombinate)	0.08 ± 0.13	35.58 ± 0.71	75.84 ± 2.18	2.132 ± 112	44	96	367-fold
IAC-enriched 226/N6 (bigenic milk)	0.66 ± 0.25	6.34 ± 0.64	22.10 ± 1.10	2.506 ± 267	< 1	63	16-fold
IAC-enriched 226/N6 (trigenic milk)	0.03 ± 0.01‡	10.32 ± 1.92	43.21 ± 2.75	4.187 ± 1.425	39	10	70-fold

NA, not applicable; rFVIII, recombinant factor VIII. *Determined by ELISA. †Determined by activated partial thromboplastin time (APTT). IAC enriched Recombinate used as a control. IAC enrichment calculated from FVIII antigen to total protein ratio.

and 226/N6 exhibited strong VWF binding signals in a similar dose-dependent fashion (Fig. 3). The similar polynomial fits to each of these absorption profiles indicated that FL-rFVIII and 226/N6 have similar avidities for VWF in human plasma.

Hemostatic potential of infused IAC-enriched 226/N6

We studied the hemostatic efficacy of 226/N6 in vivo by using a terminal tail transection bleeding model in HA mice. Figure 4 shows the median total blood loss observed for normal C57/BL mice and FVIII knockout HA mice resulting from a terminal tail transection. The HA mice were infused with LR solution, IAC-enriched 226/N6 made in CHO cells, or IAC-enriched 226/N6 from milk. 226/N6 from CHO cells and 226/N6 from transgenic milk were each infused at 80 IU kg^{-1} body weight. Normal C57/BL mice showed a cessation of bleeding (four of four) at about 2–5 min, with the total weight of blood loss over 10 min ranging from 80 to 165 mg. HA mice infused with LR solution typically did not show a cessation of bleeding (10 of 12), with the total weight of blood loss ranging from 115 to 725 mg. HA mice infused with 226/N6 from CHO cells showed a cessation of bleeding (five of five) at about 2–5 min, with the total weight of blood loss ranging from 60 to 230 mg. HA mice infused with 226/N6 from transgenic milk showed a cessation of bleeding (five of six) typically at about 2–5 min, with most mice having a total weight of blood loss ranging from 10 to 175 mg. Post hoc analysis (two-tailed *t*-tests) showed that LR solution-treated HA mice bled significantly more than LR solution-treated C57/BL mice ($P \leq 0.001$, $\alpha = 0.05$), whereas both 226/N6 from CHO cells and 226/N6 from transgenic milk-treated HA mice had similar blood losses as LR solution-treated C57/BL mice ($P = 0.574$ and $P = 0.491$, respectively, $\alpha = 0.05$).

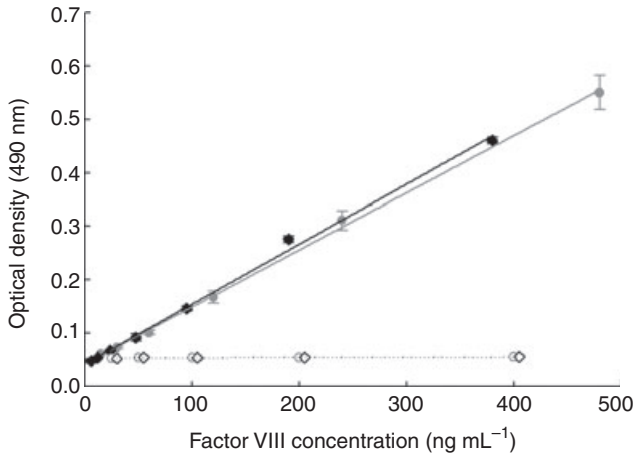

Fig. 3. Plasma von Willebrand factor (VWF) binding of immunoaffinity chromatography (IAC)-enriched 226/N6. The relative affinities of VWF for IAC-enriched 226/N6 from milk from trigenic mice (◆) and FL-rFVIII (●) were compared by ELISA. The specificity of VWF binding was also evaluated by preincubating IAC-enriched 226/N6 from milk from trigenic mice (○) and FL-rFVIII (◇) with a monoclonal antibody (BO2C11) that binds to FVIII and inhibits VWF binding. Polynomial trendlines are shown for the reference FL-rFVIII (gray line) and 226/N6 (black line) in the absence of the inhibitor, and the reference FL-rFVIII (dotted gray line) and 226/N6 (dotted black line) in the presence of the inhibitor. Error bars represent standard deviations.

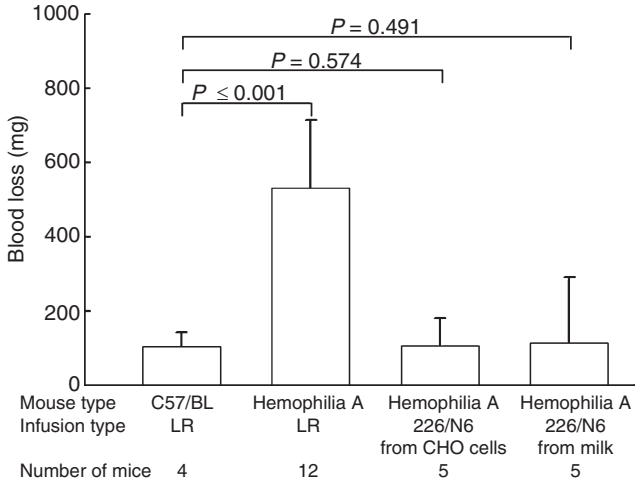

Fig. 4. Hemostatic efficacy in vivo of 226/N6 within the hemophilia A (HA) mouse model. Total blood loss (in mg) over 10 min, caused by a terminal tail transection, was observed for normal C57/BL mice and FVIII knockout HA mice. The HA mice were infused with either lactated Ringer's (LR) solution, immunoaffinity chromatography (IAC)-enriched 226/N6 from CHO cells, or IAC-enriched 226/N6 from transgenic milk. An analysis of variance indicated that the effect of treatment was significant ($P < 0.001$). Post hoc analysis showed that LR solution-treated HA mice bled significantly more than LR solution-treated control mice ($P \leq 0.001$). An α-value of 0.05 was used as a significance criterion for all statistics. Bars represent the median blood loss and the error bars the standard deviations.

Discussion

The production of rFVIII in the milk of transgenic livestock is a promising approach to significantly increase the availability of rFVIII therapy. Producing more rFVIII will help to advance immunotolerance and non-intravenous therapy [31,32]. Towards that goal, our previous work and the work of others showed that FL-rFVIII was efficiently secreted into milk at 10–1000-fold higher concentrations than are seen in blood plasma or cell culture (Table S1). However, the specific activity of the FL-rFVIII was very low. The broad range of FL-rFVIII levels expressed in milk is probably caused by transcriptional inefficiency, which often occurs as a result of transgene integration into less transcriptionally active chromosomal sites [29,30]. In founder lineages with favorable integration sites for expression, this work shows that 226/N6 can be made at high concentrations, similar to that of FL-rFVIII. Therefore, 226/N6 is at least as efficiently secreted by the mammary gland as FL-rFVIII, and additional improvements in transcriptional efficiency might enable even higher levels to be obtained. The 226/N6, which has a modified B domain lacking furin sites, yielded a specific activity similar to that of therapeutic-grade reference FL-FVIII and FL-rFVIII. Importantly, the infusion of IAC-enriched 226/N6 from milk or CHO cells corrected the hemostatic defect of FVIII knockout mice caused by a tail transection trauma challenge.

Coexpressed AAT did not appear to impact on the specific activity of the milk-borne 226/N6, as the specific activities of 226/N6 produced by monogenic, bigenic and trigenic mice were similar. AAT was added to enriched preparations to inhibit plasmin generation during storage, because plasmin is a naturally occurring, broadly acting protease in milk [33,34]. The general coexpression of milk protease inhibitors in order to protect the recombinant protein product is still a future developmental goal.

This is the first report of any rFVIII being coexpressed in milk with rVWF. FVIII circulates as a complex with VWF that is a prerequisite for its stability and thus its survival in the circulation [13,35]. Therapeutic-grade FL-FVIII:VWF complex and 226/N6 in the presence of coexpressed rVWF had similarly reduced electrophoretic mobilities. This, in addition to the similar in vitro binding of plasma-derived VWF by 226/N6, suggests that coexpressed VWF:226/N6 complexes existed in the milk. The native binding of 226/N6 with plasma-derived VWF indirectly shows that the proper post-translational sulfation of Tyr1680 in the A3 domain of 226/N6 probably occurred, as it is required for complexation [36]. 226/N6 expressed with and without rVWF in milk had > 450-fold higher activity levels than the analogous bioreactor-produced FL-rFVIII and > 40-fold higher activity levels than 226/N6 expressed in CHO cell culture without rVWF [14]. The high activity levels accumulating in the milk in the presence or absence of rVWF probably reflect the inherent stability of 226/N6. This contrasts with the beneficial effects of VWF coexpressed in cell culture, which stabilizes FL-rFVIII and increases its accumulated activity levels [13,15,16]. This study provides the first proof that VWF binds to this 226/N6 design and that a coexpressed FVIII:VWF product may be feasible in milk.

We acknowledge that the utilization of a bioengineered rFVIII could result in neoantigenicity; however, the advantage

of the 226/N6 design is that its bioengineering strategy targets modifications to the B domain, where inhibitor antibodies have infrequently been described. In addition, after thrombin activation, 226/N6 assumes an active FVIII structure that is identical to full-length wild-type FVIII [14]. There is also evidence that VWF complexation with FVIII increases the effectiveness of immune tolerance therapy [37]. Thus, the ability of 226/N6 to complex with coexpressed rVWF and plasma-derived VWF may be a beneficial attribute in the development of future applications of transgenically produced rFVIII.

The safety and efficacy of transgenic milk-derived products has crossed an important threshold, as the US Food and Drug Administration and European Medicines Agency have approved recombinant anti-thrombin III made in goat milk (ATryn, GTC Biotherapeutics, Framingham, MA, USA) [38]. Our study in mice provides the fundamental understanding needed for the future expression of biologically active, single-chain rFVIII molecules of different designs in the milk of livestock. Pigs are a likely choice of transgenic livestock for future production of rFVIII [32], because they combine the advantages of milk volume and hepatocyte-like post-translational biochemistry, such as glycosylation [39]. With respect to 226/N6, which retains six of the 19 potential glycosylation sites in the B domain, the glycosylation may impact on pharmacokinetics. Although ruminants also provide an ample milk volume, their mammary biochemistry produces higher-mannose, low sialic acid glycoforms that may affect circulation residence times, owing to interactions with asialyo receptors found in the human liver [40]. Given the fundamental knowledge about rFVIII stability provided by our studies in mice and pigs, we have begun to make transgenic pigs with various modified B domain FVIII constructions. In contrast to mice, the amount of rFVIII that will be produced in pig milk will permit bioengineering studies on purification and formulation. The advantage of pig milk over other FVIII sources, such as cell culture bioreactors and human plasma, is evident in the greater than two orders of magnitude reduction in source volume estimated to be needed to meet global FVIII demand [38] (Table S3).

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Disclosure of Conflict of Interests

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic of FL-rFVIII, 226/N6 and molecular masses of FVIII species after cleavage at furin sites.

Figure S2. Comparison of plasma-derived FVIII and FL-rFVIII heterodimers in the milk of pigs by western blot.

Figure S3. Transgene constructs and detection of monogenic, bigenic and trigenic mice made with 226/N6 (WAP7FVIII-226/N6), VWF (WAP6VWF) and A1AT (BLG-AAT) transgenes.

Figure S4. Expression of the BLG gene in trigenic mice (226/N6-AAT-rVWF) analyzed by western blot.

Figure S5. Expression of 226/N6 and VWF in trigenic mice (226/N6-AAT-rVWF) analyzed by western blot.

Figure S6. IAC enrichment of 226/N6 in low-expressing mice.

Table S1. The concentration and specific activity of FL-rFVIII in milk.

Table S2. The specific activities of therapeutic-grade FL-FVIII and FL-rFVIII and IAC-enriched 226/N6 determined with one-stage and two-stage clotting assays.

Table S3. Estimated volumes of source materials needed for current global FVIII consumption.

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