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ORIGINAL RESEARCH PAPER

Efficient stabilization of recombinant human coagulation factor VIII in the milk of transgenic mice using *hFVIII* and *vWF* co-expression vector transduction

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Abstract

Objectives   To investigate the reasons for the instability of human coagulation factor FVIII (hFVIII) in milk which is an intractable obstacle during the hFVIII production by a transgenic mammary gland bioreactor. *Results*   We constructed P1A3-hFVIIIBDD and P1A3-hFVIIIBDD-IRES-vWF co-expression cassettes for generating transgenic mice. P1A3-hFVIII/CMV-vWF double heterozygotes were also prepared by mating P1A3-hFVIIIBDD with CMV-vWF mice. hFVIII bioactivity in milk was determined under different storage conditions. The half-life (in vitro) of hFVIII bioactivity in P1A3-hFVIIIBDD-IRES-vWF mice was significantly longer than P1A3-hFVIIIBDD mice [77 ± 4.9 vs. 44 ± 2.6 h at 4 °C, 32.5 ± 5 vs. 19.7 ± 0.6 h at room temperature and 7.4 ± 1.4 vs. 3.4 ± 0.6 at 37 °C, respectively ($P < 0.05$)]. The half-life (in vitro) of hFVIII bioactivity in milk of double heterozygotes was similar to P1A3-hFVIIIBDD-IRES-vWF ones, demonstrating that the *vWF* transgene expression in hFVIII transgenic mice can efficiently improve the stabilization of hFVIII bioactivity in milk.
Conclusion   We provide a new approach of P1A3-hFVIIIBDD-IRES-vWF co-expression to generate more stable hFVIII in transgenic milk with rapid and low cost as well as valuable information for producing pharmaceutical proteins by transgenic mammary gland bioreactor.

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Abbreviations

hFVIII	Human coagulation factor VIII
hFVIIIBDD	*hFVIII* cDNA fragment with B domain deleted
IRES	Internal ribosome entry site
P1A3	Goat β-casein promoter region
vWF	von Willebrand factor

Introduction

Hemophilia A is an inherited X-linked recessive bleeding disorder caused by human coagulant factor VIII (hFVIII) deficiency or dysfunction, with an incidence of 1 in 5000 males (Farid 2007). Currently, the standard curative option for patients with severe hemophilia A is repeated infusions of hFVIII concentrates either from normal human plasma or recombinant hFVIII (Wang et al. 2013). With the advances in biotechnology of transgenic animals, transgenic mammary gland bioreactor is considered as a new production method and has great prospects to produce pharmaceutical proteins (Schmidt 2006; Wei et al. 2011). The safety and efficacy of transgenic milk-derived products has crossed an important threshold as the first recombinant protein, Atryn (recombinant human antithrombin III) produced in the milk of transgenic goats has been approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMEA) (Schmidt 2006).

hfVIII has been expressed in the milk of several animals, such as mice, rabbits, sheep, and pigs (Chen et al. 2002; Chrenek et al. 2007; Niemann et al. 1999; Paleyanda et al. 1997). Due to its large molecular weight, complex structure and instability, hFVIII degrades in transgenic milk under conventional storage conditions (Tang et al. 2013). Therefore, the maintenance of its stability is one of the key elements in the final hFVIII product.

Normally, hFVIII is closely integrated to form a complex by the light chain bonding to the von Willebrand factor (vWF) in the plasma. Expression of vWF in transgenic milk may increase the stability of hFVIII protein. The present study was to investigate whether *hFVIII* and *vWF* transgene co-expression could efficiently stabilize the bioactivity of recombinant hFVIII protein in the transgenic milk using an hFVIII and vWF co-expression vector transduction approach. Our data indicated that vWF increased the stability of hVIII in milk of the transgenic animals.

Materials and methods

Generating and screening of transgenic mice

The three vectors (P1A3-hFVIIIBDD, CMV-vWF and P1A3-hFVIIIBDD-IRES-vWF) were digested with *Sal*I (TaKaRa) and the target fragments were purified with QIA quick Gel Extraction Kit (Qiagen). The details of transgenic cassettes are given in Supporting information and Supplementary Fig. 1. Purified DNA fragments were microinjected into the pronuclear of fertilized specific pathogen free (SPF) KM mouse strain zygotes. The founder mice were mated with WT ones to obtain F1 generation and the same method to obtain heterozygous F2 and F3 generations. The P1A3-hFVIIIBDD transgenic mice were mated with CMV-vWF transgenic mice to produce double heterozygous P1A3-hFVIIIBDD/CMV-vWF transgenic mice, and PCR with tail biopsy DNA samples was used to screen the transgenic mice (see Supplementary Materials).

Real-time PCR

Genomic DNA was used for real-time PCR amplification using the primer pairs specific to the *hFVIIIBDD* (forward: 5′-CCAACATGATGGCATG-GAAG-3′; reverse: 5′-CGAGGACTAAGGGAGCA-TAG-3′) and housekeeping gene β-*actin* (forward: 5′-CTACAATGAGCTGCGTGTGG-3′; reverse: 5′-CA GGTCCAGACGCAGGATGGC-3′). Each reaction contained 250 ng genomic DNA, 1 pmol each primer, and 12.5 µl SYBR Premix Ex TaqTM Mix in a final reaction of 25 µl. The assay was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The copy number of the transgenic mice of the *hFVIII* was normalized to the β-*actin* gene.

RT-PCR (reverse transcription polymerase chain reaction)

Total RNA was isolated using TRIzol from transgenic and WT mice mammary gland tissues on day 10 of lactation. About 500 ng total RNA was reverse-transcribed to obtain cDNA with Reverse Transcriptase M-MLV (Rnase H-) and Ribonuclease Inhibitor (Takara Biotechnology, Japan) using oligo-dT16 primers. PCR was processed with the *hFVIIIBDD* cDNA specific primers (forward: 5′-TTAGTCAA-CAAAGCAGGTCC-3′; reverse: 5′-GATGAGAACC GAAGCTGGTA-3′) with a product of 1201 bp and *vWF* primers (forward: 5′-AGTCCTGCTGTCAGAC AGATAC-3′; reverse: 5′-CACACTGCCTATACTCC ATACC-3′) with a fragment of 491 bp. Mice transferrin receptor housekeeping gene (TFRC, as the

internal control) was also amplified with specific primers (forward: 5′-TGACTGCACCGGCAATTTC-3′; reverse: 5′-GGTACCCTCTGGAAGTTTAACGA A-3′) as a product of 147 bp.

Western blot detection of hFVIII or vWF

Milk samples collected from lactating transgenic and WT mice were centrifuged at $10,000\times g$ for 10 min to remove the upper lipid layer. The defatted milk samples were diluted at 1:20 by PBS and boiled at 95 °C for 5 min with SDS-PAGE loading buffer. After electrophoresis on 10 % SDS-PAGE, the proteins were transferred to PVDF transfer membrane (0.45 μm, Pierce, USA). The membranes were blocked with 3 % BSA/TBS overnight at 4 °C and then incubated with mouse monoclonal antibody to hFVIII light chain (dilution 1:1000, 3 % BSA, Clone 24-7-C7, Milliproe, USA) for 2 h at room temperature. The membranes were incubated with HRP-conjugated rabbit anti mouse IgG (dilution 1:4000, 3 % BSA, Dako, Denmark) at room temperature for 1 h. After thorough washing, the immunodetection was carried out with ECL plus western blotting substrate detection reagent A and B (32134, Thermo Scientific, Pittsburgh, USA) according to the manufacturer's instructions.

The western blot for vWF in mouse milk was processed with rabbit polyclonal antibody to human vWF (dilution 1:1000, 3 % BSA, A0082, Dako, Denmark) as first antibody, and other steps were same to the detection of hFVIII.

Immunohistochemistry (IHC)

Mammary glands of transgenic and WT mice were collected under anesthesia for IHC analyses. Serial sections, 5 μm, were fixed with acetone for 10 min at 4 °C and treated with 0.3 % Triton X-100 in PBS for 15 min at room temperature. Sections were then blocked with 10 % goat serum for 30 min at room temperature, and stained with mouse-anti-hFVIII and rabbit-anti-vWF primary antibodies (clone GMA-012, Millipore, Boston, USA; A0082, Dako, Denmark) at 4 °C overnight. These sections continued to be incubated at 37 °C for 60 min, and washed with PBS. Then, the sections were stained with secondary antibodies, namely, goat-anti-mouse IgG antibodies conjugated with fluorescein isothiocyanate (FITC) (Invitrogen) and

iFluor 594 goat anti-rabbit IgG (AAT Bioquest, USA), at 37 °C for 30 min, and washed with PBS. The nuclei were counter-stained with DAPI. The sections were analyzed with a fluorescence microscope.

Determination of hFVIII or vWF in the milk samples by ELISA

Milk samples were collected from lactating transgenic and WT mice on day 10 of lactation. The samples were diluted at 1:50 with 3 % bovine serum albumin (BSA) for ELISA, using sheep-anti-hFVIII polyclonal antibody (Abcam, Cambridge, UK) as coating antibody and mouse-anti-hFVIII monoclonal antibody (Abcam, Cambridge, UK) as primary antibody. Horse radish peroxidase (HRP)-enzyme-linked rabbit-anti-mouse antibody (P0161, Dako, Copenhagen Denmark) was used as secondary antibody with TMB Substrate Kit assay (34021, Thermo Scientific, Pittsburgh, USA). Absorption was measured by enzyme-labeled instrument (Synergy2, BioTek, Burlington, USA) at 450 nm. ELISA kit for detection of vWF was purchased from Ray Biotech (ELH—vWF, Ray Biotech, Atlanta, USA).

ELISA was also used to determine the hFVIII–VWF binding in transgenic milk. Samples were loaded into 96-well plates precoated with anti-FVIII antibody (Abcam, Cambridge, UK). Anti-VWF antibody (Dako, Denmark) was used to detect vWF binding. The absorbance was read at 450 nm.

Biological activity assay of hFVIII in the milk of transgenic mice

Activity of hFVIII in mouse milk was determined using modified APTT (activated partial thromboplastin time test) with an automatic thrombin (ACL TOP700, Instrumentation Laboratory, MA, USA). The milk samples were diluted and added the plasma with deficient factor VIII. Correction of the clotting time of the deficient plasma was proportional to the concentration (% activity) of that factor in the milk samples, interpolated from a calibration curve.

To probe the function of vWF for stability of hFVIII in the milk of transgenic mice, the hFVIII activity was assayed in milk samples from P1A3-hFVIIIBDD, P1A3-hFVIIIBDD-IRES-vWF co-expression and P1A3-hFVIIIBDD/CMV-vWF double heterozygous transgenic mouse lines at different storage conditions

(4 °C, room temperature and 37 °C, respectively) as well as various time points. Indicial plots of mean recombinant hFVIII activity for the test lots v incubation time were constructed, and the time of recombinant hFVIII bioactivity declining to the half was calculated as the half-life (in vitro) via this exponential equation analysis.

Statistical analysis

At least three repeats of each sample were carried out and the results of multiple experiments were reported as mean ± standard deviation. The t test was used to compare differences between the means of indicated groups. SPSS statistical software (SPSS, Chicago, IL, USA) was used for data analysis.

Results and discussion

We created several *hFVIII* transgenic mice. The mice with stable expression of exogenous genes in the mouse milk for at least three generations were chosen for further research. They were P1A3-hFVIIIBDD line #54, P1A3-hFVIIIBDD-IRSE-vWF co-expression line #2, CMV-vWF line #22, and also P1A3-hFVIIIBDD/CMV-vWF double heterozygous line #54/22. PCR analysis showed that the exogenous fragments were present in the genome of these transgenic mice (Supplementary Fig. 2). The copy number of exogenous genes in these transgenic mice was various, such as 28 in P1A3-hFVIIIBDD #54, 7 in P1A3-hFVIIIBDD-IRES-vWF co-expression #2 and 20 in CMV-vWF #22 determined by real-time PCR (Table 1). Meanwhile, RT-PCR analysis showed the presence of *hFVIIIBDD* or *vWF* transcripts (Fig. 1a), while western blot, IHC and ELISA analysis indicated the expression of hFVIII or vWF protein in transgenic mice (Figs. 1a, 2; Table 1), consistent with previous work (Chrenek et al. 2007; Niemann et al. 1999). The initial bioactivity of hFVIII in fresh milk from three transgenic mouse lines (P1A3-hFVIIIBDD #54, P1A3-hFVIIIBDD-IRES-vWF co-expression #2 and P1A3-hFVIIIBDD/CMV-vWF line #54/22) was also detected by APTT. Statistical analysis revealed that recombinant hFVIII activity in above three transgenic mouse lines was not significantly different ($P > 0.05$) (Table 1).

With the time extension as well as the difference of storage condition, the half-life (in vitro) of hFVIII bioactivity among these transgenic mice was diverse. The half-life (in vitro) of hFVIII bioactivity in P1A3-hFVIIIBDD-IRES-vWF mice was significantly longer than P1A3-hFVIIIBDD mice [77.2 ± 4.9 vs. 43.8 ± 2.6 h at 4 °C, 32.5 ± 5 vs. 19.7 ± 0.6 h at room temperature and 7.4 ± 1.4 vs. 3.4 ± 0.6 at 37 °C, respectively, ($P < 0.05$)]. The half-life (in vitro) of hFVIII bioactivity in milk of double heterozygotes was similar to P1A3-hFVIIIBDD-IRES-vWF ones, apparently longer than that of P1A3-hFVIIIBDD transgenic mice ($P < 0.05$). There was no statistical difference between line #54/22 and the line #2 ($P > 0.05$) (Fig. 3). The tendency of hFVIII concentration to degrade in milk was the same as observed in the same mouse lines (Supplementary Table 1). These results indicated that the *vWF* transgene expression in hFVIII transgenic mice can efficiently improve the stabilization of hFVIII in milk.

To investigate whether the stability of hFVIII in the transgenic milk resulted from the combination of hFVIII with vWF, we performed hFVIII–vWF binding ELISA experiment. The results showed that hFVIII exhibited strong vWF binding signals in a dose-dependent manner with both P1A3-hFVIII-IRES-vWF co-expression (#2) and P1A3-hFVIII/CMV-vWF double heterozygous mouse milk (#54/22), while no vWF was detected in the milk from P1A3-hFVIII mice (#54) (Supplementary Fig. 3). Our results indicated that the stability of hFVIII in transgenic milk was mainly due to its binding with vWF.

The formation of hFVIII: vWF complex is crucial to the stability of hFVIII in plasma since it prevents possible cell surface receptor-mediated decomposition (Jacquemin 2009; Lenting et al. 2010) or plasma protease-mediated hydrolysis (Fay et al. 1991). We speculate that the interaction between the hFVIII light chain and vWF makes the recombinant hFVIII more stable in the transgenic milk, and three binding regions of hFVIII were identified (Jacquemin 2009). The acidic region of the *N*-end from 1672aa to 1689aa located in the A3 domain, especially the sulfation of Tyr1680, is very important to regulate the bond with vWF (Saenko and Scandella 1997). Tang et al. (2013) produced a *hFVIIIBDD* mutant, which was incapable of binding vWF, by deleting the vWF-binding region in the A3 domain of hFVIII and the hFVIIIBDD

Table 1 Transgene copy number and exogenous protein expression in milk of the transgenic mice

Transgenic mouse line number	Transgene copy number	hFVIII concentration (μg/ml)	hFVIII clotting activity (mU/ml)	vWF concentration (μg/ml)
P1A3-hFVIIIBDD #54	28	2.73 ± 0.63	757 ± 138	–
P1A3-hFVIIIBDD-IRES-vWF #2	7	2.49 ± 0.56	785 ± 90	22.12 ± 2.30
CMV-vWF #22	20	–	–	28.3 ± 2.56
P1A3-hFVIIIBDD/CMV-vWF #54/22	28/20	2.52 ± 0.46	694 ± 79	27.36 ± 3.70
WT	–	–	–	–

No less than four females of each transgenic mouse group were respectively detected. WT (wild type) was used as control
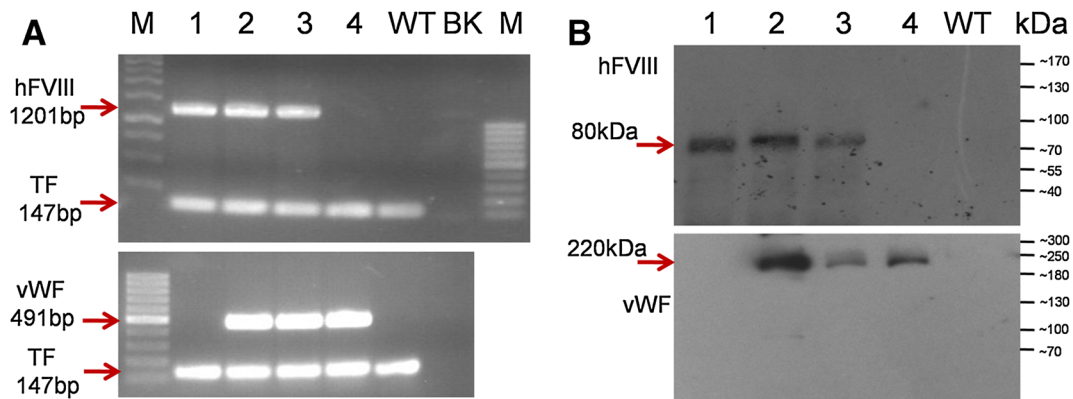

Fig. 1 Detection of hFVIII expression in the mammary gland of transgenic mice by RT-PCR and Western blot. **a** RT-PCR analysis of the *hFVIII* transcripts in transgenic mouse mammary glands. M: 1 kb or 100 bp molecular weight marker. *1–4* samples from P1A3-hFVIIIBDD, P1A3-hFVIIIBDD-IRES-vWF co-expression, P1A3-hFVIIIBDD/CMV-vWF double heterozygous and CMV-vWF transgenic mice, respectively. *WT* wild type mouse, *BC* blank. Mouse TF housekeeping gene is amplified as internal control. **b** Western blotting analysis of hFVIII and vWF in the milk. *1–4* milk samples obtained from P1A3-hFVIIIBDD line #54, P1A3-hFVIIIBDD-IRES-vWF co-expression line #2, P1A3-hFVIIIBDD/CMV-vWF double heterozygous line #54/22 and CMV-vWF line #22 transgenic mice in order. *2* and *3* show the hFVIII light chain (80 kDa) and the vWF subunit (220 kDa) simultaneously, while *1* represents hFVIII and *4* is vWF solely. WT stands for wild type mouse

mutant was expressed in cells. Pharmacokinetic studies in *FVIII* knockout mice by the same group showed that the terminal half-life ($T_{1/2}$) of hFVIIIBDD mutant was dramatically reduced relative to hFVIIIBDD (0.6 vs. 6 h). In addition, the hFVIII:vWF complex possibly inhibits plasmin generation during storage, because plasmin is a naturally-occurring, broad-acting protease in milk (Ismail and Nielsen 2010).

Pipe et al. (2011) reported that vWF could play a role in increasing of recombinant hFVIII bioactivity in transgenic milk by mating hFVIII mice with vWF mice. They produced various hFVIIIBDD transgenic mice (hFVIII remained 226aa of B domain with six glycosylation site, as 226/N6) including monogenic mice (226/N6), bigenic mice (226/N6-BLG-AAT) and trigenic mice (226/N6-vWF-BLG-AAT). Detection of hFVIII proved that 226/N6 expressed with vWF in milk had sixfold higher activities than that without vWF forming the hFVIII:vWF complex. However, the effect of vWF on hFVIII stability was not elucidated in the transgenic mouse milk. Particularly, the preparation of double heterozygous animals was time-consuming, laborious and segregation of genetic characters in the actual operation, while the effect for stabilizing hFVIII of P1A3-hFVIIIBDD/CMV-
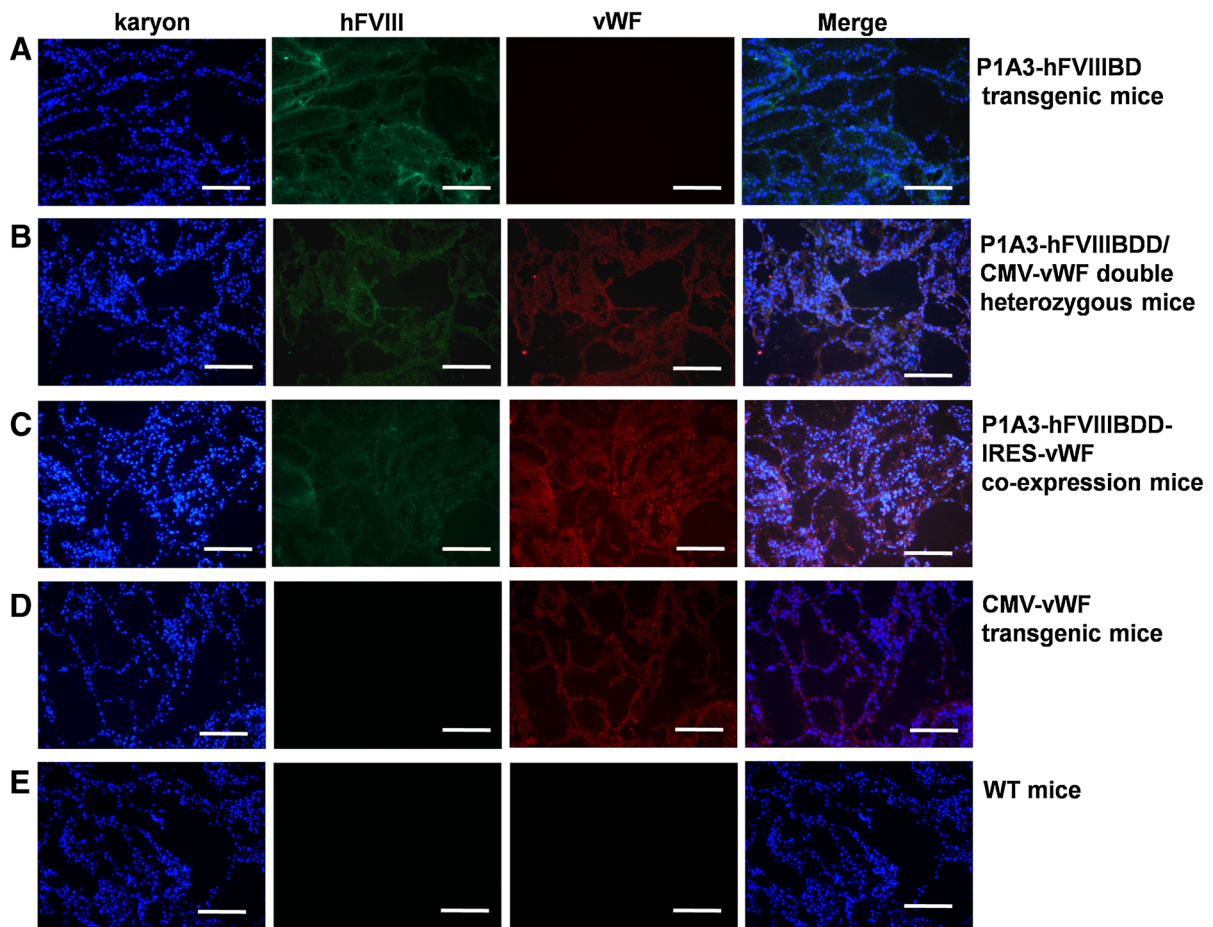
Fig. 2 Immunohistochemistry analyses of hFVIII and vWF proteins in mammary gland tissues. P1A3-hFVIIIBDD, P1A3-hFVIIIBDD-IRES-vWF co-expression, P1A3-hFVIIIBDD/CMV-vWF double heterozygous, CMV-vWF transgenic mice and wild type mice are sacrificed, and 5-μm serial cryosections are stained with specific anti-hFVIII antibodies or/and anti-vWF antibodies, followed by examination under the microscopy. The sections are stained positively for hFVIII (*green*) or vWF (*red*). **a** P1A3-hFVIIIBDD transgenic mouse shows the expression of hFVIII protein in the section examined. **b** P1A3-hFVIIIBDD/CMV-vWF double heterozygous transgenic mouse shows the simultaneous expression of hFVIII and vWF proteins. **c** P1A3-hFVIIIBDD-IRES-vWF co-expression transgenic mouse also shows both the hFVIII and vWF proteins expressed in the sections examined. **d** CMV-vWF transgenic mouse shows the expression of vWF protein, no hFVIII positive staining. **e** No specific staining is observed in WT mice. *Scale bar* is 50 μm. Original magnifications: ×200

vWF heterozygous mice was not better than that of P1A3-hFVIII-IRES-vWF co-expression mice (Fig. 3; Supplementary Table 1). Thus, this approach was likely not applicable for the hFVIII transgenic mammary gland bioreactor. Our study performed the stability determination of hFVIII bioactivity in transgenic milk at various times under different storage conditions and indicated that the co-expression of hFVIII and vWF efficiently increased the stability of hFVIII in transgenic milk in vitro. Furthermore, the construction of P1A3-hFVIIIBDD-IRES-vWF co-

expression cassette for generating transgenic animals would be more valuable and low cost for the production of hFVIII transgenic mammary gland bioreactor.

Conclusion

This study provides a new approach to produce a more stable hFVIII protein in transgenic milk by using *hFVIII* and *vWF* transgene co-expression vectors. It can defer hFVIII degradation during transportation,
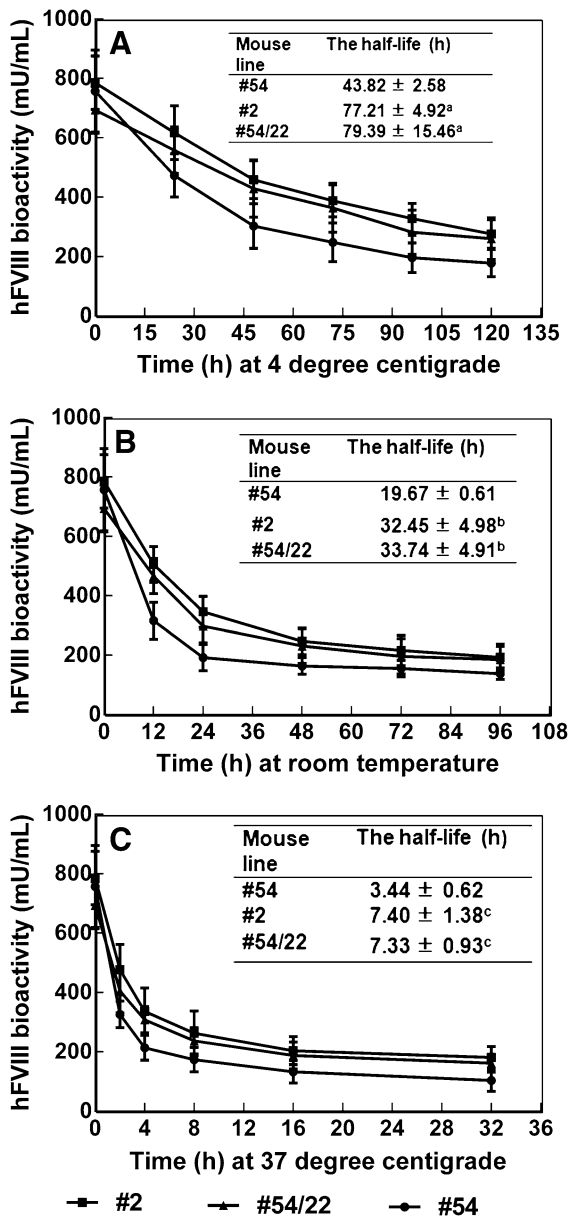
Mouse line	The half-life (h)
#54	43.82 ± 2.58[a]
#2	77.21 ± 4.92[a]
#54/22	79.39 ± 15.46[a]

A

Mouse line	The half-life (h)
#54	19.67 ± 0.61
#2	32.45 ± 4.98[b]
#54/22	33.74 ± 4.91[b]

B

Mouse line	The half-life (h)
#54	3.44 ± 0.62
#2	7.40 ± 1.38[c]
#54/22	7.33 ± 0.93[c]

C

◄Fig. 3 The vWF extends the half-life (in vitro) of hFVIII in the milk of transgenic mice. The *line chart* shows the changes of hFVIII bioactivity (ordinate) with the delay of time (*abscissa*). The average half-life (in vitro) was calculated by indicial plots of recombinant hFVIII activity for the test lots v incubation time. The *inset* table presents the mean half-time of recombinant hFVIII activity for each tested temperature. At all storage conditions, the half-time of hFVIII bioactivity in P1A3-hFVIIIBDD line #2 is significantly shorter than other two ($P < 0.05$). Simultaneously, the half-life (in vitro) of hFVIII bioactivity is no significantly difference between P1A3-hFVIIIBDD-IRES-vWF co-expression line #2 and P1A3-hFVIIIBDD/CMV-vWF double heterozygous line #54/22 ($P > 0.05$). (N ≥ 4) *Superscript lower-case letters* indicate significant differences compared to P1A3-hFVIIIBDD line #54 ($P < 0.05$)

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handling and storage procedures. This information will be valuable for the production of pharmaceutical proteins using transgenic mammary gland bioreactors.

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