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PRODUCTION OF RECOMBINANT HUMAN COAGULATION FACTOR IX BY
TRANSGENIC PIG

by

Weijie Xu

A DISSERTATION

Presented to the Faculty of

The Graduation College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Chemical and Biomolecular Engineering

Under the Supervision of Professor William H. Velander

Lincoln, Nebraska

July, 2014

PRODUCTION OF RECOMBINANT HUMAN COAGULATION FACTOR IX BY TRANSGENIC PIG

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University of Nebraska, 2014

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Hemophilia B is the congenital bleeding disorder caused by deficiency in functional coagulation factor IX (FIX) and about 28,000 patients worldwide in 2012. And current treatment is restricted to protein-replacement therapy, which required FIX concentrates for patients' life-time. Approximately 1 billion units FIX were consumed in 2012. However, still about 70-80% patients, mostly in developing countries, received inadequate or no treatment because of the unavailable and/or unaffordable FIX concentrates. Considering safety reasons, e.g. transmission of blood-borne diseases, the recombinant human FIX (rFIX) is recommended other than the plasma-derived FIX. However, only one rFIX is currently available on the market. The complexity of the FIX protein and its post-translational modifications (PTMs) cause the limit quantity and unaffordable high price of the recombinant human FIX. We previously reported successfully expressing recombinant human FIX in the milk by mammary gland of transgenic pig (tg-FIX) and established a lab-scale purification protocol to achieve active tg-FIX. The expression of tg-FIX level was about 2-3 g/L and with 10-20% specific activity. The final purified high acidic tg-FIX had the specific activity closed to the normal human plasma derived FIX. In this study, efforts were aim to further increase the yield of the active tg-FIX from the transgenic pig. First, we investigated

degradation/activation of the tg-FIX in the milk by predominant milk-borne protease, plasmin. This provided us the data in decreasing the degradation and activation of final products. Then, to process the excessive non-active pro-peptide attached tg-FIX, we bio-engineered the mammary gland by introducing truncated human furin gene. The co-expressed recombinant furin cleaved pro-peptide of tg-FIX both in mammary epithelium, it also secreted and cleaved the pro-peptide in milk. This bio-engineering has been proved not interfering the over-expression of the tg-FIX and its specific activity. Finally, to mass producing active tg-FIX, we established the large scale purification protocol. The purified active tg-FIX was achieved within four chromatography steps with full specific activity. Not like the previous one, the active tg-FIX was further enriched and the contaminant activated tg-FIX was totally eliminated. This study made progress in producing recombinant human FIX economically.

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CHAPTER 1: INTRODUCTION

BACKGROUND

Coagulation factor IX and Coagulation Cascade

Coagulation Factor IX (FIX) gene is located on the X-chromosome at q27. Gene is expressed in hepatocytes with a highly tissue-specific manner as a pre-pro form (Fig.1) (1,2). The primary structure of FIX protein consist 40 amino acids γ -carboxyglutamate (Gla) domain, two Epidermal Growth Factor (EGF) domains (a.a. 47-127), activation peptide (a.a. 146-180) and the catalytic domain (a.a. 181-415). The first two domains compose the light chain (Mr. ~20 kDa) and the catalytic domain composes the heavy chain (Mr. ~28 kDa), in which His-221, Asp-269 and Ser-365 compose the serine protease catalytic triad. The nascent FIX is proceeded by several post translational modifications (PTM)s (Fig. 1), including the removal signal peptide, vitamin-K dependent gamma-carboxylation of first 12 glutamic acid(3,4), pro-peptide removal(5,6), glycosylation(7-10), beta-hydroxylation of Asp-64(11), sulfation of Tyr-155(12) and phosphorylation of Ser-158(13-15).

At the physiologic concentrations of Ca^{2+} (1.1 mM) and Mg^{2+} (0.6 mM), the core Gla domain of Vitamin K dependent (VKD) coagulation proteins like Protein C, Factor VII, Factor X and prothrombin have 4 Ca^{2+} and 2 Mg^{2+} specific sites and also 1 binding site that can accommodate either divalent cation. The FIX has 2 additional Gla than other VKD factors and one more Mg^{2+} specific sites. Binding of above cations to the Gla domain is required for its conformational rearrangement from a disordered form to an ordered and organized form(16,17); This conformational rearrangement is essential for

factor IX to bind to negatively charged phospholipid vesicles provided *in vivo* by activated platelets resulting in its local ionization and augmentation of activation(18). Gla domain also interacts with the C-terminal domain of TF in the TF/FVIIa complex during the FIX activation (19). In addition, the it is essential for FX activation by binding of FVIIIa, co-factor of tenase complex(20).

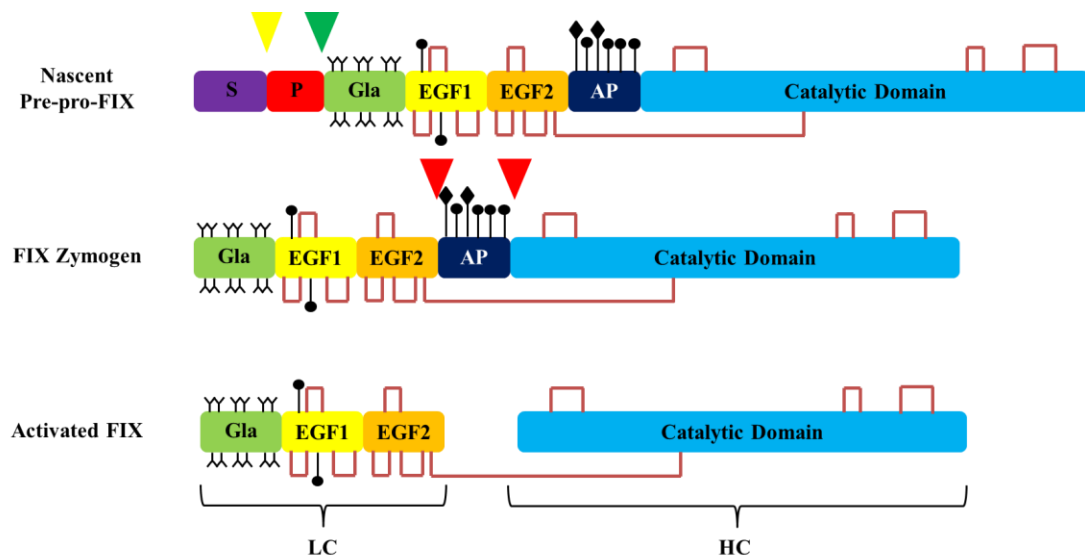


Fig.1. The Primary Structure of Nascent FIX, Zymogen, Activated FIX and their PTMs. S: signal Peptide; P: pro-peptide; Gla: γ -carboxylglutamate domain; EGF: epithelium growth factor domain; AP: activation peptide; ∇ : signal peptide cleavage site; ∇ : pro-peptide cleavage site; Y: γ -carboxylglutamates; \uparrow : O-link glycans; \uparrow : N-link glycans; \square : disulfide bonds; ∇ : activation cleavage sites.

The mature FIX secrets and circulates as a 55-57kDa zymogen form in the blood at a physiological concentration of 5 μ g/ml (21). When tissue damage occurs, the blood coagulation cascade is initiated when the tissue factor (TF) comes in contact with activated factor VII (FVIIa). The TF-FVIIa complex initiates the coagulation cascade and finally forms the insoluble clot of activated platelets and fibrin polymers. Thrombin generated in the extrinsic pathway also activates the intrinsic pathway of blood

coagulation (Fig. 2) (22). Bauer, et. al. reported that FIXa generated *in vivo* mainly from the activity of the TF-FVIIa rather than the intrinsic pathway(23).

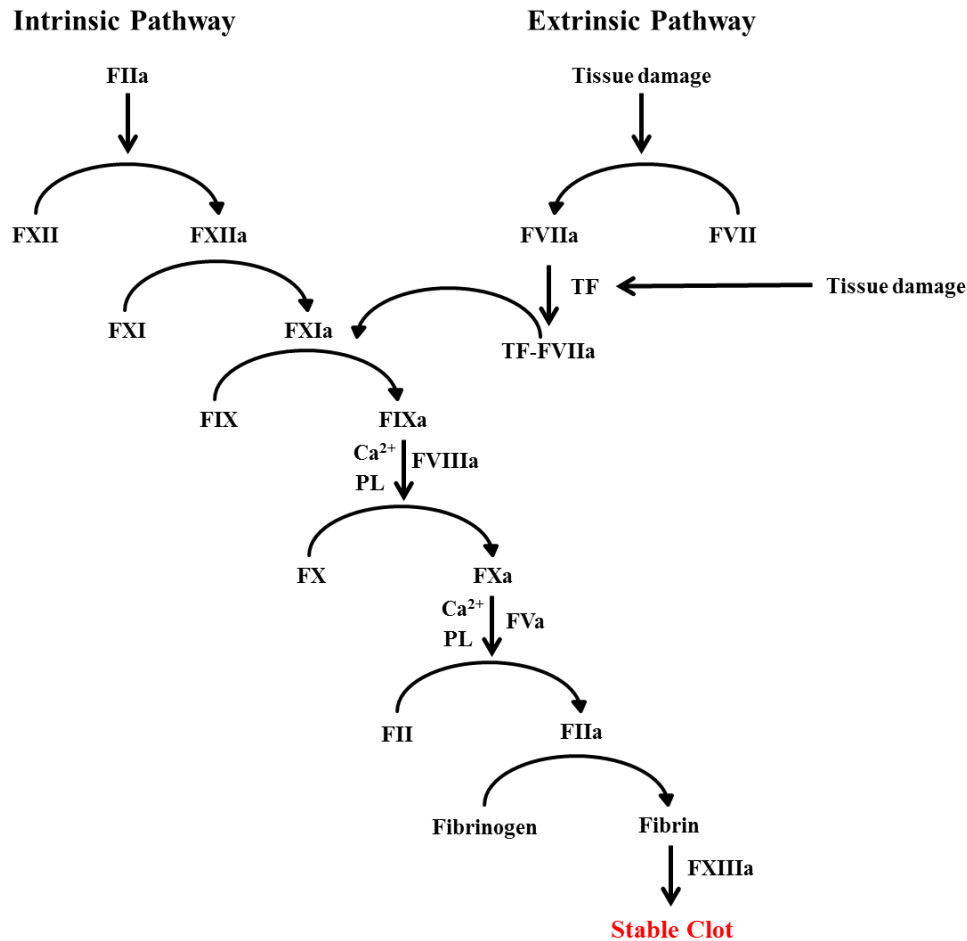


Fig. 2. The Coagulation Cascade. (Modified from Davie, E.W., et. al. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* 30, 10363-70 (1991).

Role of Pro-peptide in γ -carboxylation and Pro-peptide Processing

Furie et. al. reported the deletion of the pro-peptide (residues -18 to -1) inhibiting vitamin K dependent carboxylation(24). Study of *in vitro* carboxylation indicated the carboxylase recognition sites was the pro-peptide (25,26). And in the study of pro-peptides between Vitamin K dependent proteins, the amino acids sequence showed highly homological at

Phe⁻¹⁶ and Ala⁻¹⁰. Both mutation in pro-peptide, Phe⁻¹⁶ to Ala and Ala⁻¹⁰ to Glu completely inhibited the carboxylation (27,28). It is indicated that the role of pro-peptide in carboxylation, and the order in PTMs, the removal of pro-peptide should follow the gamma-carboxylation.

In addition, homology is shown in basic amino acids at residues -4, -2 and -1(29,30). Both FIX Cambridge (Arg⁻¹ to Ser) and FIX San Dimas (Arg⁻⁴ to Glu) are partially carboxylated and having pro-peptide attached hemophilia FIXs (31,32). In studies of other hemophilia FIXs with the mutation in Arg⁻⁴ to Gln (FIX Seattle C), Leu (FIX Bendorf) or Trp (FIX Boxtel), although mutations do not affect gamma-carboxylation, the defective propeptide cleavage results in destabilizing the calcium-induced Gla domain conformation change and no pro-coagulation activity of pro-peptide attached FIX (33). Furin or paired basic amino acid cleaving enzyme (PACE), a Ca²⁺ dependent serine protease of the PC family, cleaves pro-peptides at dibasic sites with consensus sequence Arg-X-Lys/Arg-Arg (RXK/RR)(34). Furin undergoes an auto-activation, the 83-residue N-terminal propeptide is autoproteolytically excised in the endoplasmic reticulum (ER) at the consensus furin site, -Arg¹⁰⁴-Thr-Lys-Arg¹⁰⁷-. Then, the propeptide attached furin transport to the acidified TGN/endosomal compartments, furin cleaves the bound propeptide at P1/P6 Arg site (-Arg⁷⁰-Gly-Val-Thr-Lys-Arg⁷⁵-) resulting in propeptide dissociation and enzyme activation(35). The mature furin is localized primarily to the trans-Golgi network (TGN)(36-39), and also traffics between TGN and cell surface(40,41). The previous study also indicated that ER retention of furin blocked its activation resulting in no bioactivity. On the other hand, the vitamin K-dependent carboxylase is an integral membrane protein associated with endoplasmic reticulum(24).

The order in location of carboxylase (ER) and furin (TGN) *in vivo* assure the pro-peptide removal after the carboxylation formed.

Hemophilia B and Protein Replacement Treatments

Hemophilia B is the congenital bleeding disorders and is caused by deficiency in functional coagulation factor IX (FIX). Various mutations in FIX gene can impair the pro-coagulation activity of the FIX protein, resulting in bleeding-disorder(42). Since this an X-linked recessive chromosomal disorder, almost all Hemophilia B patients are male, 25,850 (92%) patients were male; only 719 (3%) patients were female (43). In absence or abnormal level of functional FIX, both the extrinsic and intrinsic coagulation pathway cannot be accomplished, patients experience frequent and recurrent bleeding episodes. Soft tissue hemorrhages and intracranial bleeding, potential to cause long-lasting damage to internal organs and can be life-threatening in some instances(44). Recurrent bleeding episodes are also associated with a range of longer-term clinical consequences, including musculoskeletal problems, which have the potential to restrict functional impairment in later life (45). The degree of impact associated with hemophilia is determined by the severity of the condition (46,47). Approximately 60–70% of hemophilia B patients have a moderate (defined as clotting factor concentration 0.05–0.40 IU/ml, experience prolonged bleeding responses to relatively minor trauma) or severe (<0.01 IU/ml, experience frequent spontaneous bleeds) form of the condition(48-50). Current treatment of hemophilia B is restricted to protein-replacement therapy, which centers on two distinct approaches: on-demand treatment and prophylaxis. The on-demand treatment offers short-term and immediate compensation of FIX to stop further hemorrhaging and minimize the impact of a bleeding episode. Conversely, prophylaxis focus upon

maintaining acceptable levels of baseline FIX, which decrease the frequency of acute hemorrhage and life-threatening bleeding episodes and is associated with better long-term outcomes (e.g. preventing joint damage and arthropathy)(51-53). In 2012, approximately 250 million units of FIX concentrate was consumed in U.S., and about 1 billion units worldwide(43). Currently, there are 15 FIX concentrates on the market (Table 1). 14 of them are plasma derived (pd-) FIX, although the purification protocols was improved in virus removal over the last 20 years, there are still some concerns regarding prions and non-capsulated viruses (54,55). Only one is recombinant (r-) human FIX (BeneFIX), which was introduced by 1988 to the market(56), and it has been proven to be safe and effective with low incidence of serious adverse effects(57).

Brand Name	Company	Source	Fractionation	Viral Inactivation	Specific Activity (IU/mg)
Aimafix®	Kedrion	Plasma	Anion exchange, DEAESephadex®/Sepharose®, Heparin-affinity chromatography	TNBP/polysorbate 80; dry heat, 100°C for 30 min; nanofiltration, 35 + 15 nm	>100
AlphaNine® SD	Grifols	Plasma	Dual polysaccharide ligand affinity chromatography	S/D; TNBP/polysorbate 80; nanofiltration	210
Berinin® P	CSL Behring	Plasma	DEAE Sephadex, Heparin-affinity chromatography	Pasteurization at 60°C for 10 hours	146
Betafact®	LFB	Plasma	Ion exchange, affinity chromatography	TNBP/polysorbate 80; nanofiltration, 15 nm	110
Factor IX Grifols®	Grifols	Plasma	Precipitation, Multiple chromatography	S/D; nanofiltration, 15 nm	>150
Haemonine®	Biotest	Plasma	Anion exchange, Immunoaffinity, Hydrophobic–interaction chromatography	TNBP/polysorbate 80; nanofiltration, 15 nm	>70
Hemo-B-RAAS	Shanghai RAAS	Plasma	Ion exchange, Affinity chromatography	S/D; dry heat; nanofiltration	>50
Immunine®	Baxter BioScience	Plasma	Ion exchange, Hydrophobic–interaction chromatography	Polysorbate 80; vapor heating, 60°C for 10 hours; 190 mbar, then 80°C for 1 hour, 375 mbar	~100
Mononine®	CSL Behring	Plasma	Immunoaffinity chromatography	Sodium thiocyanate; ultrafiltration	>190

Brand Name	Company	Source	Fractionation	Viral Inactivation	Specific Activity (IU/mg)
Nanotiv®	Octapharma	Plasma	Ion exchange, Affinity chromatography	TNBP/Triton® × 100; nanofiltration	>150
Nonafact®	Sanquin	Plasma	Immunoaffinity, Hydrophobic-interaction chromatography	TNBP/polysorbate 80; nanofiltration, 15 nm	≥200
Octanine F®	Octapharma	Plasma	Ion exchange, Affinity chromatography	TNBP/polysorbate 80; nanofiltration	>120
Replenine®-VF	Bio products laboratory	Plasma	Metal chelate chromatography	S/D; nanofiltration, 15 nm	200
TBSF FIX	CSL biotherapies	Plasma	Ion exchange, Heparin-affinity chromatography	TNBP/polysorbate 80; nanofiltration	>50
BeneFIX®	Pfizer	Recombinant	Anion chromatography	Nanofiltration	≥200

Table 1. Factor IX Concentrates on Market (Modified from Franchini, M., et. al. Treatment of hemophilia B: focus on recombinant factor IX. *Biologics* 7, 33-8 (2013)). Abbreviations: DEAE, diethylaminoethanol; S/D, solvent–detergent; TNBP, tri-n-butyl phosphate.

Although the in vivo activity recovery of r-FIX lower than the pd-FIX results in 1.5 to 2-fold increasing in r-FIX dosing(58,59), considering bio-safety reasons, the application of r-FIX products is widely recommended(60) In United States, 99% of consumed FIX concentrate was recombinant, while only 59% worldwide in 2012(43). The FIX concentrates account for up to 98% of the total cost of hemophilia care(61). Mean annual coagulation factor costs for on-demand treatment of haemophilia (A and B) ranging from € 24,771 (\$34,184) in the United Kingdom to €92,918 (\$128,227) in Germany, ranging from €112,727 (\$155,563) in Netherlands to €182,075 (\$251,264) in Germany for prophylaxis(62). Approximately 80% patients, mostly in the developing countries, received inadequate or no treatment because of the limited supplies and high costs of either r- or pathogen screened, pd-FIX concentrates(63,64).

Recombinant Therapeutics Production and Transgenic Livestock

The first therapeutic protein was the porcine insulin in 1920s, and recombinant human insulin prepared in bacteria was introduced in the market by early 1980s. By using the recombinant therapeutics, no more contamination of blood-borne pathogen needs to be worried about. Because of the complexity of the human protein and their *in vivo* post-translational modifications (PTMs), the bacteria had its limitation in producing recombinant proteins. Then, yeast, insect cells, mammalian cells, transgenic plants and animals were developed in producing complex therapeutics, which could not be produced correctly by bacteria. The Chinese Hamster Ovary (CHO) cells were widely used in current industrial scale manufacturing. All these therapeutic bioreactor systems has their advantages and limitations (Table 2)

Points to consider	Production systems					
	Bacteria	Yeast	Insect cells	Animal cells (CHO cells)	Transgenic plasnts	Transgenic animals
Practical production level	++ (+)	++ (+)	+	+	++	++++
Investment cost	+++++	+++++	++	+	++++	+++
Production cost	+++++	+++++	++	++	+++++	++++
Line stability	+++++	+++++	++++	+++	+++++	+++++
Scaling up	+++++	+++++	++	+	+++++	++++
Collection	+++++	+++++	+++++	+++++	+++++	++++
Post-translational modifications	+	++	+++	++++	+++	++++
Stability of product	+++++	+++++	+++	+++	++++	++++
Purification	+++	+++	+++	++++	+++	+++

Table 2. Comparison of the different systems to produce recombinant pharmaceutical proteins. (Modified from Houdebine, L. M. Production of pharmaceutical proteins by transgenic animals. 2009.)

Transgenic Livestock in Producing Recombinant Therapeutics

In 1985, Hammer and colleagues established the first transgenic livestock animals(65). Since then, more and more pharmaceutical recombinant proteins were produced by using the transgenic animals (66-69). Mammary gland is currently the best available bioreactor

among all tissues in transgenic animals (66,68). The cell density of the gland is about 10^9 cell mL⁻¹, which could be 2-3 orders of magnitude greater than density of mammalian cells culture (64); the milk production, pig as example is about 200-400 liters per lactation with 1-2 lactations per year; and more important, the mammary gland of transgenic animals could provide certain PTMs, like signal and pro-peptide removal, sulphation, glycosylation, carboxylation, which highly relate to the activity and recovery *in vivo*, to the recombinant proteins. And the transgenic pig was successfully in producing recombinant human PC(70,71), fibrinogen(72), FVIII(73) and FIX(74) in the milk.

The most successful example, ATryn, human antithrombin, is made from the milk of transgenic goats and approved by FDA in 2009 for treatment of patients with hereditary antithrombin deficiency(75). Only one transgenic goat produced same amount of antithrombin in a year as 90,000 blood donations (76). The cost and easy scaling up of therapeutics production is also the advantage of the transgenic livestock bioreactor. A previous study suggested that building a large-scale (10,000 liter bioreactor) manufacturing facility for mammalian cells takes 3–5 years and costs US\$ 250–500 million, whereas a transgenic farm with a single purification facility should not cost more than US\$80 million and would most likely cost less (66). So that, using the mammary gland of transgenic livestock as the bioreactor to express recombinant human FIX (tg-FIX) could be a promising source to produce FIX concentrates in a safe and more economical way. However, because of the complexity of the PTMs of human FIX, no all transgenic livestock are suitable for manufacturing this recombinant therapeutics. No active FIX had been detected in the transgenic sheep milk(77). Zhang et al reported

that >90% recombinant protein in milk appeared to be a gamma-glycosylated and biologically active but the expression level was only 13.7 ng/mL(78).

Current Progress and Specific Aims

Our lab has generated transgenic pigs that express high level (2-3 g/l) transgenic FIX (tg-FIX) in the milk, and also developed the protocol for purifying active tg-FIX by using the combination of heparin-affinity chromatography (whole tg-FIX population) and anion exchange (Mini Q) chromatography (high acidic/carboxylated tg-FIX subpopulation); the final purified tg-FIX had the specific activity at 197 U/mg(74). This tg-FIX was characterized in extent of γ -carboxylglutamate and N-glycosylation. Not like the plasma-derived human FIX, the tg-FIX has different extent of carboxylation subpopulations. At tg-FIX expression level was at 200 μ g/L milk, it was highly carboxylated (≥ 10) (79). However, when the expression level increased about 1,000-fold (2 mg/ml), only 10-20% was highly carboxylated (74), this indicated the enzymatic PTMs were rate limited and block the yield of the active tg-FIX production. Van Cott et. al. reported the majority of the N-glycans of this tg-FIX were complex, bi-antennary, while pd-FIX N-glycans were highly branched (tri- and tetra-antennary), and highly sialylated. No detectable Neu5Gc nor Gal α (1,3)Gal in tg-FIX, which is strongly antigenic in human(80). We also noticed, at this high tg-FIX expression level, approximately 10-30% of total tg-FIX population is pro-tgFIX (pro-peptide attached tg-FIX) that has no pro-coagulation activity. In addition, we noticed certain amount of degraded/activated tg-FIX in the high acidic tg-FIX subpopulation.

In this study, we were working on the following specific aims:

Specific Aim#1: Characterize the tg-FIX and its degraded species in purified high acidic tg-FIX subpopulation. And investigate the predominant milk-borne protease, plasmin, in degrading the tg-FIX and the extent of the Gla in protecting FIX being cleaved.

Specific Aim#2: Bioengineer pig mammary gland by introducing truncated human furin gene co-expressing with tg-FIX. Characterize the recombinant truncated human furin (r-furin) in processing the excessive pro-peptide and convert non-active tg-proFIX to active tg-FIX both *in vivo* and *in vitro*.

Specific Aim#3: Establish scaled-up purification protocol, using the more efficient, immunoaffinity chromatography FIX select column to capture whole tg-FIX population from transgenic milk; then enrich high acidic tg-FIX by anion exchange chromatography; finally isolate the active tg-FIX subpopulation by using HPLC-SEC.

REFERENCE

1. Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W., and Kurachi, K. (1985) Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* **24**, 3736-3750
2. Kurachi, K., and Kurachi, S. (1995) Regulatory mechanism of the factor IX gene. *Thromb Haemost* **73**, 333-339
3. Burnier, J. P., Borowski, M., Furie, B. C., and Furie, B. (1981) Gamma-carboxyglutamic acid. *Mol Cell Biochem* **39**, 191-207
4. Morris, D. P., Stevens, R. D., Wright, D. J., and Stafford, D. W. (1995) Processive post-translational modification. Vitamin K-dependent carboxylation of a peptide substrate. *J Biol Chem* **270**, 30491-30498
5. Bravo, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) Accurate and efficient cleavage of the human insulin proreceptor by the human proprotein-processing protease furin. Characterization and kinetic parameters using the purified, secreted soluble protease expressed by a recombinant baculovirus. *J Biol Chem* **269**, 25830-25837
6. Steiner, D. F. (1998) The proprotein convertases. *Curr Opin Chem Biol* **2**, 31-39
7. Nishimura, H., Kawabata, S., Kisiel, W., Hase, S., Ikenaka, T., Takao, T., Shimonishi, Y., and Iwanaga, S. (1989) Identification of a disaccharide (Xyl-Glc) and a trisaccharide (Xyl₂-Glc) O-glycosidically linked to a serine residue in the

- first epidermal growth factor-like domain of human factors VII and IX and protein Z and bovine protein Z. *J Biol Chem* **264**, 20320-20325
8. Nishimura, H., Takao, T., Hase, S., Shimonishi, Y., and Iwanaga, S. (1992) Human factor IX has a tetrasaccharide O-glycosidically linked to serine 61 through the fucose residue. *J Biol Chem* **267**, 17520-17525
 9. Harris, R. J., van Halbeek, H., Glushka, J., Basa, L. J., Ling, V. T., Smith, K. J., and Spellman, M. W. (1993) Identification and structural analysis of the tetrasaccharide NeuAc α (2 \rightarrow 6)Gal β (1 \rightarrow 4)GlcNAc β (1 \rightarrow 3)Fuc α 1 \rightarrow O-linked to serine 61 of human factor IX. *Biochemistry* **32**, 6539-6547
 10. Agarwala, K. L., Kawabata, S., Takao, T., Murata, H., Shimonishi, Y., Nishimura, H., and Iwanaga, S. (1994) Activation peptide of human factor IX has oligosaccharides O-glycosidically linked to threonine residues at 159 and 169. *Biochemistry* **33**, 5167-5171
 11. Fernlund, P., and Stenflo, J. (1983) Beta-hydroxyaspartic acid in vitamin K-dependent proteins. *J Biol Chem* **258**, 12509-12512
 12. Kaufman, R. J. (1998) Post-translational modifications required for coagulation factor secretion and function. *Thromb Haemost* **79**, 1068-1079
 13. Atoda, H., Yokota, E., and Morita, T. (2006) Characterization of a monoclonal antibody B1 that recognizes phosphorylated Ser-158 in the activation peptide region of human coagulation factor IX. *J Biol Chem* **281**, 9314-9320
 14. White, G. C., 2nd, Beebe, A., and Nielsen, B. (1997) Recombinant factor IX. *Thromb Haemost* **78**, 261-265
 15. Arruda, V. R., Hagstrom, J. N., Deitch, J., Heiman-Patterson, T., Camire, R. M., Chu, K., Fields, P. A., Herzog, R. W., Couto, L. B., Larson, P. J., and High, K. A. (2001) Posttranslational modifications of recombinant myotube-synthesized human factor IX. *Blood* **97**, 130-138
 16. Tulinsky, A., Park, C. H., and Skrzypczak-Jankun, E. (1988) Structure of prothrombin fragment 1 refined at 2.8 Å resolution. *J Mol Biol* **202**, 885-901
 17. Soriano-Garcia, M., Park, C. H., Tulinsky, A., Ravichandran, K. G., and Skrzypczak-Jankun, E. (1989) Structure of Ca²⁺ prothrombin fragment 1 including the conformation of the Gla domain. *Biochemistry* **28**, 6805-6810
 18. Liebman, H. A., Furie, B. C., and Furie, B. (1987) The factor IX phospholipid-binding site is required for calcium-dependent activation of factor IX by factor XIa. *J Biol Chem* **262**, 7605-7612
 19. Kirchhofer, D., Lipari, M. T., Moran, P., Eigenbrot, C., and Kelley, R. F. (2000) The tissue factor region that interacts with substrates factor IX and Factor X. *Biochemistry* **39**, 7380-7387
 20. Blostein, M. D., Furie, B. C., Rajotte, I., and Furie, B. (2003) The Gla domain of factor IXa binds to factor VIIIa in the tenase complex. *J Biol Chem* **278**, 31297-31302
 21. Enfield, D. L., and Thompson, A. R. (1984) Cleavage and activation of human factor IX by serine proteases. *Blood* **64**, 821-831
 22. Davie, E. W., Fujikawa, K., and Kiesel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* **30**, 10363-10370
 23. Bauer, K. A., Kass, B. L., ten Cate, H., Hawiger, J. J., and Rosenberg, R. D. (1990) Factor IX is activated in vivo by the tissue factor mechanism. *Blood* **76**, 731-736

24. Furie, B., and Furie, B. C. (1991) Molecular basis of gamma-carboxylation. Role of the propeptide in the vitamin K-dependent proteins. *Ann N Y Acad Sci* **614**, 1-10
25. Ulrich, M. M., Furie, B., Jacobs, M. R., Vermeer, C., and Furie, B. C. (1988) Vitamin K-dependent carboxylation. A synthetic peptide based upon the gamma-carboxylation recognition site sequence of the prothrombin propeptide is an active substrate for the carboxylase in vitro. *J Biol Chem* **263**, 9697-9702
26. Hubbard, B. R., Jacobs, M., Ulrich, M. M., Walsh, C., Furie, B., and Furie, B. C. (1989) Vitamin K-dependent carboxylation. In vitro modification of synthetic peptides containing the gamma-carboxylation recognition site. *J Biol Chem* **264**, 14145-14150
27. Tai, M. M., Furie, B. C., and Furie, B. (1980) Conformation-specific antibodies directed against the bovine prothrombin . calcium complex. *J Biol Chem* **255**, 2790-2795
28. Rabiet, M. J., Jorgensen, M. J., Furie, B., and Furie, B. C. (1987) Effect of propeptide mutations on post-translational processing of factor IX. Evidence that beta-hydroxylation and gamma-carboxylation are independent events. *J Biol Chem* **262**, 14895-14898
29. Price, P. A., and Williamson, M. K. (1985) Primary structure of bovine matrix Gla protein, a new vitamin K-dependent bone protein. *J Biol Chem* **260**, 14971-14975
30. Price, P. A., Fraser, J. D., and Metz-Virca, G. (1987) Molecular cloning of matrix Gla protein: implications for substrate recognition by the vitamin K-dependent gamma-carboxylase. *Proc Natl Acad Sci U S A* **84**, 8335-8339
31. Diuguid, D. L., Rabiet, M. J., Furie, B. C., Liebman, H. A., and Furie, B. (1986) Molecular basis of hemophilia B: a defective enzyme due to an unprocessed propeptide is caused by a point mutation in the factor IX precursor. *Proc Natl Acad Sci U S A* **83**, 5803-5807
32. Ware, J., Diuguid, D. L., Liebman, H. A., Rabiet, M. J., Kasper, C. K., Furie, B. C., Furie, B., and Stafford, D. W. (1989) Factor IX San Dimas. Substitution of glutamine for Arg-4 in the propeptide leads to incomplete gamma-carboxylation and altered phospholipid binding properties. *J Biol Chem* **264**, 11401-11406
33. Wojcik, E. G., Van Den Berg, M., Poort, S. R., and Bertina, R. M. (1997) Modification of the N-terminus of human factor IX by defective propeptide cleavage or acetylation results in a destabilized calcium-induced conformation: effects on phospholipid binding and activation by factor XIa. *Biochem J* **323** (Pt 3), 629-636
34. Hatsuzawa, K., Hosaka, M., Nakagawa, T., Nagase, M., Shoda, A., Murakami, K., and Nakayama, K. (1990) Structure and expression of mouse furin, a yeast Kex2-related protease. Lack of processing of coexpressed prorenin in GH4C1 cells. *The Journal of biological chemistry* **265**, 22075-22078
35. Anderson, E. D., Molloy, S. S., Jean, F., Fei, H., Shimamura, S., and Thomas, G. (2002) The ordered and compartment-specific autoproteolytic removal of the furin intramolecular chaperone is required for enzyme activation. *The Journal of biological chemistry* **277**, 12879-12890

36. Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E., and Thomas, G. (1994) Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *The EMBO journal* **13**, 18-33
37. Voorhees, P., Deignan, E., van Donselaar, E., Humphrey, J., Marks, M. S., Peters, P. J., and Bonifacino, J. S. (1995) An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface. *The EMBO journal* **14**, 4961-4975
38. Schafer, W., Stroh, A., Berghofer, S., Seiler, J., Vey, M., Kruse, M. L., Kern, H. F., Klenk, H. D., and Garten, W. (1995) Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. *The EMBO journal* **14**, 2424-2435
39. Jones, B. G., Thomas, L., Molloy, S. S., Thulin, C. D., Fry, M. D., Walsh, K. A., and Thomas, G. (1995) Intracellular trafficking of furin is modulated by the phosphorylation state of a casein kinase II site in its cytoplasmic tail. *The EMBO journal* **14**, 5869-5883
40. Wan, L., Molloy, S. S., Thomas, L., Liu, G., Xiang, Y., Rybak, S. L., and Thomas, G. (1998) PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization. *Cell* **94**, 205-216
41. Molloy, S. S., Thomas, L., Kamibayashi, C., Mumby, M. C., and Thomas, G. (1998) Regulation of endosome sorting by a specific PP2A isoform. *The Journal of cell biology* **142**, 1399-1411
42. Green P.M., G. F., Sommer S.S., Poon M.-C., Ludwig M., Schwaab R., Reitsma P.H., Goossens M., Yoshioka A., Figueiredo M.S., et al. . (2004) The Haemophilia B Mutation Database – version 13
<http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html>.
43. WFH. (2013) WFH Report on the Annual Global Survey 2012 Available at <http://www1.wfh.org/publications/files/pdf-1574.pdf>
44. Cavendish, M. (2008) Diseases and disorders. **2**
45. Sherry, D. D. (2008) Avoiding the impact of musculoskeletal pain on quality of life in children with hemophilia. *Orthopaedic nursing / National Association of Orthopaedic Nurses* **27**, 103-108; quiz 109-110
46. Janco, R. L., Maclean, W. E., Perrin, J. M., and Gortmaker, S. L. (1996) A prospective study of patterns of bleeding in boys with haemophilia. *Haemophilia* **2**, 202-206
47. White, G. C., Rosendaal, F., Aledort, L. M., Lusher, J. M., Rothschild, C., Ingerslev, J., and Subcommittee, F. V. F. I. (2001) Definitions in hemophilia - Recommendation of the Scientific Subcommittee on factor VIII and factor IX of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemostasis* **85**, 560-560
48. Aznar, J. A., Lucia, F., Abad-Franch, L., Jimenez-Yuste, V., Perez, R., Batlle, J., Balda, I., Parra, R., and Cortina, V. R. (2009) Haemophilia in Spain. *Haemophilia* **15**, 665-675
49. Katz, J. (1996) Prevalence of factor IX inhibitors among patients with haemophilia B: Results of a large-scale North American survey. *Haemophilia* **2**, 28-31

50. Nerich, V., Tissot, E., Faradji, A., Demesmay, K., Bertrand, M. A., Lorenzini, J. L., Briquel, M. E., Pouzol, P., and Woronoff-Lemsi, M. C. (2008) Cost-of-illness study of severe haemophilia A and B in five French haemophilia treatment centres. *Pharm World Sci* **30**, 287-292
51. Lofqvist, T., Nilsson, I. M., Berntorp, E., and Pettersson, H. (1997) Haemophilia prophylaxis in young patients - a long-term follow-up. *Journal of Internal Medicine* **241**, 395-400
52. Nilsson, I. M., Berntorp, E., Lofqvist, T., and Pettersson, H. (1992) 25 Years Experience of Prophylactic Treatment in Severe Hemophilia-a and Hemophilia-B. *Journal of Internal Medicine* **232**, 25-32
53. Manco-Johnson, M. J., and Riske, B. (2007) Prophylaxis vs. episodic treatment to prevent joint disease in severe hemophilia - Reply. *New Engl J Med* **357**, 2087-2088
54. Sharp, C. P., Lail, A., Donfield, S., Gomperts, E. D., and Simmonds, P. (2012) Virologic and clinical features of primary infection with human parvovirus 4 in subjects with hemophilia: frequent transmission by virally inactivated clotting factor concentrates. *Transfusion* **52**, 1482-1489
55. Peden, A., McCardle, L., Head, M. W., Love, S., Ward, H. J., Cousens, S. N., Keeling, D. M., Millar, C. M., Hill, F. G., and Ironside, J. W. (2010) Variant CJD infection in the spleen of a neurologically asymptomatic UK adult patient with haemophilia. *Haemophilia* **16**, 296-304
56. BeneFIX[package insert]. Philadelphia, PA: Wyeth Pharmaceuticals Inc.; 2011.
57. Franchini, M., Frattini, F., Crestani, S., Sissa, C., and Bonfanti, C. (2013) Treatment of hemophilia B: focus on recombinant factor IX. *Biologics : targets & therapy* **7**, 33-38
58. Ewenstein, B. M., Joist, J. H., Shapiro, A. D., Hofstra, T. C., Leissinger, C. A., Seremetis, S. V., Broder, M., Mueller-Velten, G., Schwartz, B. A., and Mononine Comparison Study, G. (2002) Pharmacokinetic analysis of plasma-derived and recombinant F IX concentrates in previously treated patients with moderate or severe hemophilia B. *Transfusion* **42**, 190-197
59. Bjorkman, S. (2011) A commentary on the differences in pharmacokinetics between recombinant and plasma-derived factor IX and their implications for dosing. *Haemophilia* **17**, 179-184
60. UKHCDO. (2003) Guidelines on the selection and use of therapeutic products to treat haemophilia and other hereditary bleeding disorders. *Haemophilia : the official journal of the World Federation of Hemophilia* **9**, 1-23
61. Gater, A., Thomson, T. A., and Strandberg-Larsen, M. (2011) Haemophilia B: impact on patients and economic burden of disease. *Thromb Haemost* **106**, 398-404
62. Lippert, B., Berger, K., Berntorp, E., Giangrande, P., van den Berg, M., Schramm, W., Siebert, U., and European Haemophilia Economic Study, G. (2005) Cost effectiveness of haemophilia treatment: a cross-national assessment. *Blood Coagul Fibrinolysis* **16**, 477-485
63. Stonebraker, J. S., Bolton-Maggs, P. H. B., Brooker, M., Farrugia, A., and Srivastava, A. (2011) A study of reported factor IX use around the world. *Haemophilia* **17**, 446-455

64. Van Cott, K. E., Monahan, P. E., Nichols, T. C., and Velander, W. H. (2004) Haemophilic factors produced by transgenic livestock: abundance that can enable alternative therapies worldwide. *Haemophilia* **10 Suppl 4**, 70-76
65. Hammer, R. E., Pursel, V. G., Rexroad, C. E., Jr., Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D., and Brinster, R. L. (1985) Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315**, 680-683
66. Dyck, M. K., Lacroix, D., Pothier, F., and Sirard, M. A. (2003) Making recombinant proteins in animals--different systems, different applications. *Trends in biotechnology* **21**, 394-399
67. Rudolph, N. S. (1999) Biopharmaceutical production in transgenic livestock. *Trends in biotechnology* **17**, 367-374
68. Houdebine, L. M. (2009) Production of pharmaceutical proteins by transgenic animals. *Comparative immunology, microbiology and infectious diseases* **32**, 107-121
69. Niemann, H., and Kues, W. A. (2003) Application of transgenesis in livestock for agriculture and biomedicine. *Animal reproduction science* **79**, 291-317
70. Van Cott, K. E., Lubon, H., Russell, C. G., Butler, S. P., Gwazdauskas, F. C., Knight, J., Drohan, W. N., and Velander, W. H. (1997) Phenotypic and genotypic stability of multiple lines of transgenic pigs expressing recombinant human protein C. *Transgenic research* **6**, 203-212
71. Velander, W. H., Johnson, J. L., Page, R. L., Russell, C. G., Subramanian, A., Wilkins, T. D., Gwazdauskas, F. C., Pittius, C., and Drohan, W. N. (1992) High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc Natl Acad Sci U S A* **89**, 12003-12007
72. Butler, S. P., van Cott, K., Subramanian, A., Gwazduaskas, F. C., and Velander, W. H. (1997) Current progress in the production of recombinant human fibrinogen in the milk of transgenic animals. *Thromb Haemost* **78**, 537-542
73. Paleyanda, R. K., Velander, W. H., Lee, T. K., Scandella, D. H., Gwazdauskas, F. C., Knight, J. W., Hoyer, L. W., Drohan, W. N., and Lubon, H. (1997) Transgenic pigs produce functional human factor VIII in milk. *Nature biotechnology* **15**, 971-975
74. Lindsay, M., Gil, G. C., Cadiz, A., Velander, W. H., Zhang, C., and Van Cott, K. E. (2004) Purification of recombinant DNA-derived factor IX produced in transgenic pig milk and fractionation of active and inactive subpopulations. *J Chromatogr A* **1026**, 149-157
75. Kling, J. (2009) First US approval for a transgenic animal drug. *Nature biotechnology* **27**, 302-304
76. Adiguzel, C., Iqbal, O., Demir, M., and Fareed, J. (2009) European Community and US-FDA Approval of Recombinant Human Antithrombin Produced in Genetically Altered Goats. *Clin Appl Thromb-Hem* **15**, 645-651
77. Clark, A. J., Bessos, H., Bishop, J. O., Brown, P., Harris, S., Lathe, R., Mcclenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B. A., and Wilmut, I. (1989) Expression of Human Anti-Hemophilic Factor-Ix in the Milk of Transgenic Sheep. *Bio-Technol* **7**, 487-492

78. Zhang, K., Lu, D., Xue, J., Huang, Y., and Huang, S. (1997) Construction of mammary gland-specific expression vectors for human clotting factor IX and its secretory expression in goat milk. *Chinese journal of biotechnology* **13**, 271-276
79. Van Cott, K. E., Butler, S. P., Russell, C. G., Subramanian, A., Lubon, H., Gwazdauskas, F. C., Knight, J., Drohan, W. N., and Velander, W. H. (1999) Transgenic pigs as bioreactors: a comparison of gamma-carboxylation of glutamic acid in recombinant human protein C and factor IX by the mammary gland. *Genet Anal* **15**, 155-160
80. Gil, G. C., Velander, W. H., and Van Cott, K. E. (2008) Analysis of the N-glycans of recombinant human Factor IX purified from transgenic pig milk. *Glycobiology* **18**, 526-539

CHAPER 2:

PLASMIN PROTEOLYSIS OF RECOMBINANT HUMAN FACTOR IX MADE IN THE MILK OF TRANSGENIC PIGS

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ABSTRACT

The milk-borne plasmin, identical to the one in the plasma, is the predominant protease in the milk. Producing of recombinant human coagulation factor IX (tg-rhFIX) in milk by mammary gland of transgenic pig could also be proteolyzed by the plasmin as native milk borne protein. The previous study of proteolysis of plasma-derived (pd-) human FIX by pd-plasmin showed the yield of the activated and/or degraded tg-rhFIX. Tg-rhFIX expressed in the milk frequently has subpopulations with less than a full complement (12) of γ -carboxylated glutamic acid (Gla), which alters the divalent metal-dependant conformation of the Gla domain. We noticed the cleavage of tg-rhFIX was altered by the extent of γ -carboxylated in tg-rhFIX in the presence of Ca^{2+} . The low Gla content ($\leq 10/12$) tg-rhFIX subpopulations were frequently cleaved in the light chain at Gln⁴⁴ but not in tg-rhFIX with high Gla content ($\geq 10/12$). This cleavage was also seen in the tg-rhFIX with high Gla content ($\geq 10/12$) in a chelated environment. The conformation rearrangement of high extent Gla induced by Ca^{2+} prevented the Gla domain being cleaved by plasmin.

Key words: factor IX, γ -carboxyglutamic acid, plasmin, proteolysis

INTRODUCTION

Factor IX (FIX) is a member of the vitamin K dependent family of proteins and a 56 kDa zymogen form of the serine protease activated FIX (FIXa) that contains several divalent metal binding domains including an amino-terminal region consisting of 12 γ -carboxyglutamic acid residues (Gla). At the physiologic concentrations of 1.1 mM Ca^{2+}

and 0.6 mM Mg^{2+} , the core Gla domain of Vitamin K dependent (VKD) coagulation proteins like Protein C, Factor VII, Factor X and prothrombin have 4 Ca^{2+} and 2 Mg^{2+} specific sites and also 1 binding site that can accommodate either divalent cation. The FIX has 2 additional Gla than other VKD factors and has one more Mg^{2+} specific sites. Recombinant versions of FIX (rh-FIX) frequently contain subpopulations with less than 12 Gla residues (1,2). The difference in extent of γ -carboxylation causes subpopulations with a range of biological activity. The metal-dependent conformation of the Gla domain is essential for factor IX to bind to negatively charged phospholipid vesicles provided in vivo by activated platelets resulting in its local ionization and augmentation of activation (3) and interacts with the C-terminal domain of TF in the TF/FVIIa complex during the FIX activation (4), in addition, is essential for FX activation by binding of FVIIIa, co-factor of tenase complex(5). Thus, the Gla domain plays a large role in the nature of proteolysis that occurs with zymogen FIX and other VKD proteins by serine proteases that regulate coagulation.

Plasmin is the major source of protease activity in milk (6). Furthermore, plasmin and plasminogen in milk are essentially identical to those found in blood having similar heat and pH stabilities, pH optima for casein hydrolysis, inhibitor susceptibility (7-9) and primary structure (10). Human plasma derived plasmin has been shown to proteolyze FIX into four products having apparent molecular weights of 45 kDa, 30 kDa, 20 kDa and 14 kDa when analyzed by reduced SDS-PAGE and two products at 52 kDa and 14 kDa under non-reducing conditions (11). Sequence analysis demonstrated that cleavage occurred after Lys^{43} , Arg^{145} , Arg^{180} , Lys^{316} and Arg^{318} . The conformational specificity of plasmin on FIX structure was indicated by the proteolysis at only 5 of the possible 43

plasmin sites within pd-FIX (12). The divalent metal induced conformational dependence of proteolysis (13) by plasmin has been shown in fully carboxylated pd-FIX but not partially carboxylated, biologically active rFIX. Plasmin proteolysis of FIX during circulation is normally inhibited by α_2 -antiplasmin inhibitor (14).

We previously reported producing recombinant human FIX (tg-rhFIX) in the mammary gland of transgenic pig, this expression level was about 2-3 g/L in the milk (15). This contrasts previous studies on the expression of tg-rhFIX in the milk of sheep which was largely inactive and had low levels of Gla content and appeared proteolyzed (16). Like that of recombinant FIX made by CHO cells (CHO-rhFIX), which contains different extent of Gla subpopulations (2), we fractionated tg-rhFIX into active and inactive subpopulations based upon differences in Gla content. As human and porcine plasminogen closely related (17,18), we here study the proteolysis of tg-rhFIX, therapeutic grade CHO-rhFIX and fully carboxylated pd-hFIX by human plasma derived plasmin. We also compare this proteolysis to that found in tg-rhFIX made in milk. In contrast to previous proteolysis studies of pd-FIX, we report on the differential proteolysis by plasmin to tg-rhFIX with different extent of Gla.

MATERIALS AND METHODS

All buffer components were purchased from VWR International LLC (Radnor, PA) or Thermo Fisher scientific (Waltham, MA) or Sigma (St. Louis, MO) unless otherwise stated. Reference pd-FIX (Mononine, CSL Behring, USA) and CHO-rhFIX (BeneFIX, Pfizer Limited USA) were kind gifts from James Brown (Lincoln, NE). These stocks of

were expired for clinical use but exhibited full procoagulant activity by one stage clotting assay.

Purifying High Acidic Tg-FIX from Transgenic Pig Milk

The purification procedures were adapted from previous described (15). The collected milk was adjusted to 100 mM EDTA to dissolve the casein micelle, which recover more tg-rhFIX, and stored at -80°C. The milk was thawed and then centrifuged (12,800 X g for 20 minutes) to remove the insoluble fat contained in an upper layer and solid particulates that were contained in the lower layer. The decanted middle layer or clarified milk was mixed with 4 parts 20 mM Tris, 50 mM NaCl, pH 7.4. The diluted, clarified milk was then loaded onto a Heparin sepharose 6 Fast Flow (GE Healthcare Bio-Sciences AB, Sweden) column, 10 mm I.D. X 150 mm L (11.8 mL), with the flow rate at 150 ml/hr. Then the column was washed with 20 mM Tris, 200 mM NaCl, pH 7.4 to remove caseins then with 20 mM Tris, 500 mM NaCl, pH 7.4 to elute FIX. The eluate (EL) was exchanged into the equilibrium buffer (20mM potassium phosphate, pH 7.2) and loaded on the Ceramic Hydroxyapatite (CHT) (Macro-Prep Ceramic Hydroxyapatite Type II, 40 µm, Bio-Rad Laboratories, Inc., Hercules, CA) column, 10 mm I.D. X 150 mm L (11.8 mL), with the flow rate at 150 ml/hr. The tg-FIX was eluted with a linear gradient from 20 mM to 400 mM Sodium phosphate, pH 7.2. Latter portion (about 15%) of CHT eluate (EL) was collected. The purifications were performed on a BioCAD Vision chromatography station (Applied Biosystems, Grand Island, NY). In order to minimize degradation, purification processes were performed at 4°C.

Analyzing High Acidic FIX population by High Performance/Pressure Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC)

The high acidic tg-rhFIX was exchanged into SEC running buffer (20 mM Tris, 200 mM NaCl, 10 mM CaCl₂, pH 7.4) by using the Minimate tangential flow filtration systems with 10 kDa molecular weight cut off (MWCO) membrane (Pall Corporation, Port Washington, NY) or Amicon tube (10 kDa MWCO) (EMD Millipore, Billerica, MA). Tg-FIX sample was loaded on a TSK gel G3000SWXL, 7.8 mm ID x 30 cm, 5 µm (particle size), equipped with a guard column, 6 mm ID x 4 cm, 7 µm (particle size), (Tosoh Bioscience, LLC). Columns was equilibrated and run with 20 mM Tris base, 200 mM NaCl, 10 mM CaCl₂, pH 7.0. The flow rate was 0.5 ml per min and duration of the run was 45 min with all major peaks eluting before 35 min. The fractions of each peak were pooled together for further analysis.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using precast 12% Bis-Tris Novex acrylamide gels. The gels, 4x sample buffer, 10x reducing agent, molecular weight marker and 20x running buffer were all from Invitrogen (Carlsbad, CA). Samples were incubated with reducing or non-reducing sample buffer at 70 °C for 10 minutes prior to electrophoresis at 200 V using the Invitrogen XCell Surelock mini cell. Gels were stained with Colloidal Blue (Invitrogen).

Detecting Pig Plasmin in Transgenic Milk by Western Blot

Both human plasma derived (pd-) plasmin and plasminogen were purchased from Haematologic Technologies Inc. (Essex Junction, VT). And all milk samples were skimmed and loaded on the SDS-PAGE as described and then gels were electro-blotted onto poly vinylidene fluoride (PVDF) membranes using a BioRad Transblot SemiDry

Transfer Cell (Richmond, CA) for 30 min at 25 volts. For detecting both plasmin and plasminogen, the primary antibody, Chicken IgY anti-pig plasminogen (MBS560144, MyBioSource, Inc. San Diego, CA) and secondary antibody, Goat anti-Chicken IgY-HRP (SA114013, Thermo Scientific, Rockford, IL), were used. The chromogenic DAB substrate (Thermo, Rockford, IL) was used in color developing.

NH₂-terminal Sequencing of the Plasmin Cleavage Products of FIX

Amino acid sequencing was performed at the University of Nebraska Medical Center, Protein Structure Core Facility. Protein samples were subjected to SDS-PAGE and transferred to PVDF membranes as described above. The membrane was stained with Colloidal Blue and bands excised and subjected to Edman degradation using Applied Biosystems Procise protein sequencer, a Hitachi 8800 Amino Acid Analyzer and a Michrom MAGIC HPLC equipped with a Diode Array Detector.

Quantitation of Total Factor IX Activity

Total FIX activity was determined using the activated partial thromboplastin time (aPTT) assay (19). Briefly, 50 μ l each of PTT Automate 5 reagent (Diagnostics Stago, Inc., Parsippany, NJ), factor IX deficient plasma (George King Bio-Medical, Overland Park, Kansas, USA), and a sample of interest were added to a cuvette and incubated at 37 °C for 3 min. 50 μ L of 25 mM CaCl₂ (Sigma) was then added and the clotting time was measured using the STart Hemostasis Analyzer (Diagnostics Stago, Inc., Parsippany, NJ). The normal human plasma (Diagnostics Stago, Inc., Parsippany, NJ) was used as the standard, assigning 1 unit of FIX clotting activity per milliliter of plasma. The concentration of purified tg-rhFIX was determined by measuring the optical density at 280 nm in a 1 cm quartz cuvette and using an extinction coefficient, $\epsilon^{1\%} = 13.4$.

Mass Spectrometry of Tg-rhFIX to Detect Gla Content

All tg-rhFIX samples were activated with Factor XIa (Haemtech, Essex Junction, VT) in order to separate the light chain domain containing the Gla domain from the heavy chain domain using a 1:100 (w:w) enzyme to substrate ratio in 5mM CaCl₂, 1X TBS, pH 7.4 at 37°C for 1.0 hour. After activation, the samples were quenched with 1.2 moles of EDTA per mole of calcium and stored at -80°C until further analysis. LC-ESI-TOF-MS analysis was performed on an Agilent 1200 capLC system with an Agilent 6210 ESI-TOF MS. Solvent A was 0.1% formic acid (Fluka) (v/v) in deionized water. Solvent B was 0.1% formic acid (v/v) in acetonitrile (Burdick and Jackson). The column was an Agilent 300SB-C8 Poroshell column: 7.5 cm L x 0.5 mm ID, 5 micron particle size. The samples were diluted in Solvent A in the HPLC vial and loaded in the autosampler. Approximately 35 pmol of sample was injected on the column in a 40 microliter injection volume. The column was pre-equilibrated with 5%B; the flow rate was 20 microliters/minute; the column oven was set at 37°C; the autosampler was set at 10°C. After injection, the column was washed for 5 minutes with 5%B, then a linear gradient up to 20%B over 10 minutes, followed by a linear gradient up to 55%B over 70 minutes. The column was then cleaned by ramping up to 95%B for 5 minutes and then a series of three cycles of 10%B to 70% B, and then the column was re-equilibrated for >10 CV with 0%B for the next injection. A blank injection was performed in between each sample. MS data were acquired with MassHunter in positive mode with the following parameters: 4000 V source voltage, 325°C nebulizing gas temperature, 7 L/min gas flow rate, internal reference mass of 922.01 m/z. MS data were analyzed using Agilent's Qualitative Analysis (version B.01.03).

Activating FIX to Activated FIX by FXIa

CHO-rhFIX (BeneFIX, Pfizer Limited USA) and tg-rhFIX were activated to their activated forms by plasma derived human FXIa (Haematologic Technologies, Inc., Vermont, USA). The mass ratio of FIX to FXIa is 100:1 (w:w), and both were incubated in TBS (20 mM Tris, 0.15 M NaCl) pH 7.4 with 5 mM CaCl₂ at 37°C for 2 hours. The reaction samples were exchanged into SEC running buffer and loaded on the HPLC-SEC as above. The fractions of FIXa was collected and pooled.

Plasmin Digestion of FIX and Activated FIX

FIX samples, including pd-FIX (Mononine, CSL Behring, USA), CHO-rhFIX (BeneFIX, Pfizer Limited USA) and high/low Gla tg-rhFIX, and activated FIX samples, including pd-FIXa (Haematologic Technologies, Inc., Vermont, USA), CHO-rhFIXa and tg-rhFIXa, were used in the assay. FIX and FIXa samples were incubated with plasma derived human plasmin (Haematologic Technologies, Inc., Vermont, USA) at 37°C using a 1:100 (w:w) enzyme to substrate ratio in TBS (20 mM Tris, 0.15 M NaCl, pH 7.4) in the presence of 5 mM CaCl₂ or 10 mM EDTA. Aliquots were taken out at time = 0, 10, 20, 40, 60, and 120 minutes and quenched with SDS sample loading buffer with reducing buffer (Invitrogen, Carlsbad, CA, USA), then analyzed by reduced and non-reduced SDS-PAGE. In addition, a negative control digest was analyzed with each sample by replacing the enzyme with water.

RESULTS AND DISCUSSION

Detecting Porcine Plasmin in Transgenic Milk

Transgenic milk samples, K75, K101, R175&179, R180 and R1014 lactation pools, were diluted and skimmed then loaded on the SDS-PAGE with the positive controls, human plasma derived (pd-) plasmin (83 kDa) and plasminogen (88 kDa). The antibody probed the plasmin as well. The ~ 83 kDa bands, shown in all transgenic milk samples, were porcine plasmin involving in proteolysis of tg-FIX in the transgenic milk. The amount of plasmin is about 2 μ g (compared to the pd-plasmin) in 10 μ L diluted milk sample, which has about 10 μ g tg-FIX (Fig 1).

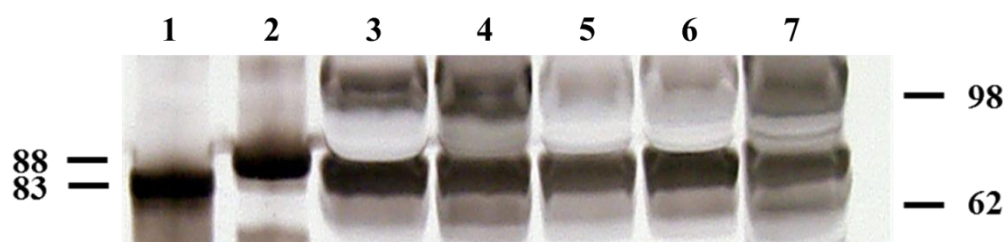


Figure 1. Non-reduced Western blot detecting porcine plasmin in the transgenic milk sample. Lane 1, human plasmin (83 kDa), 1 μ g; lane 2, human plasminogen (88 kDa), 1 μ g; lane 3-7, transgenic milk K75, K101, R175&179, R180 and R1014 lactation pool, diluted 1:1 (v/v) and skimmed, 10 μ L.

State of Degradation of Biologically Active Tg-rhFIX Pool Isolated from Milk

We previously reported that only 10-20% of the total tg-rhFIX in the milk has FIX specific activity due to rate limitations in γ -carboxylation of the Gla domain (15). We quantitatively captured these biologically active subpopulations as a pool using a sequence of heparin affinity and Ceramic Hydroxyapatite (CHT) chromatography. The high acidic tg-rhFIX obtained from CHT column contained all of the specific activity

present in the clarified whey. Conversely, the low acidic tg-rhFIX contained no biologically active species.

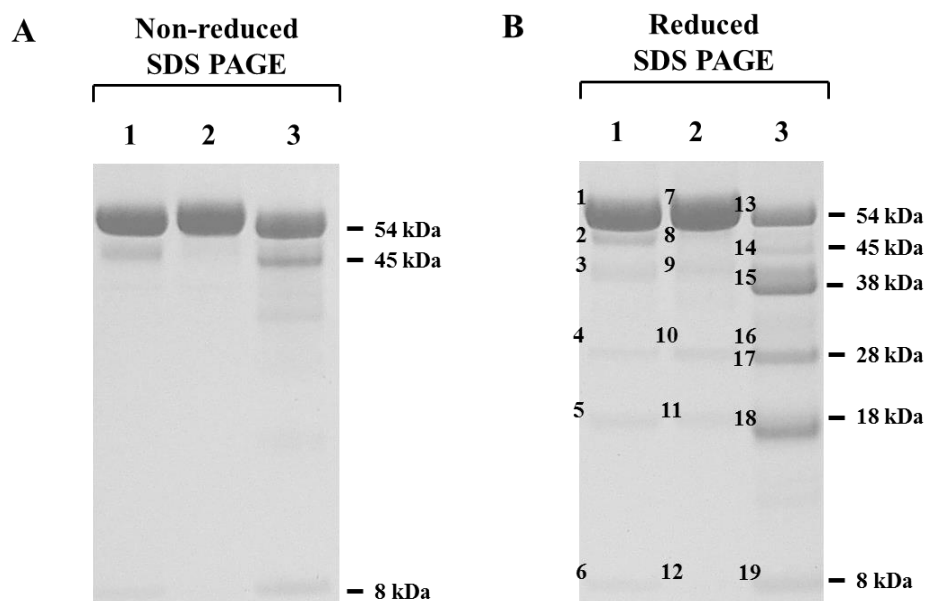


Figure 2. SDS PAGE of most acidic tg-rhFIX pool from CHT chromatography. Panel A. Non-reduced and Panel B. Reduced SDS-PAGE of FIX samples. Lane 1. pd-FIX reference; Lane 2. CHO-rhFIX reference; Lane 3. tg-rhFIX CHT most acidic pool. Each lane contains 7 μ g of protein sample. The tg-rhFIX was purified from pig K108 milk pool from lactation days 16-24.

Figure 2A compares a non-reduced Commassie Blue stained SDS-PAGE gel profile of the biologically active tg-rhFIX pool from CHT to highly purified, therapeutic grades of pd-hFIX and CHO-rhFIX. On the non-reduced SDS-PAGE, the majority of the tg-rhFIX appeared as a single band corresponding to the co-migration of both single chain and disulfide bridge-linked, two-chain species. The most common minor band in all samples was the proteolysis product termed FIX γ that results from a cleavage at Arg³¹⁸ and release of an 8 kDa carboxy-terminal peptide from the FIX parent (12). FIX γ and the released γ -peptide were present in all preparations. The MW of FIX and FIX γ species

present in pd-FIX and CHO-rhFIX were similar due to similar glycoforms and other post-translational modifications (2), but were about 2-3 kDa larger than the respective species found in tg-rhFIX. This might be due to the presence of quarternary and ternary N-linked glycoforms on the activation peptide of pd-FIX and CHO-rhFIX versus the smaller bianternary glycoforms observed in tg-rhFIX (20).

Reduced SDS-PAGE reveals the spectrum of two chain proteolytic products that were present in all FIX preparations where at least five bands appear in both reference and tg-rhFIX samples (Figure 2B). Other than the zymogen form of tg-FIX (MW 54 kDa), the predominant tg-rhFIX degraded species was FIX α , MW is about 40 to 42 kDa (Figure 2B Lane 3), which results from the cleavage at Arg¹⁴⁵ and release of the light chain (LC) from the heavy chain (HC) attaching activation peptide (AP) (FIX α -HC). This intermediate of activated FIX (FIXa), which had additional cleavage at Arg¹⁸⁰ and release of the AP, is still a biological active form of FIX if the light chain was properly carboxylated and the propeptide was removed (21).

Proteolytic Cleavages Detected by NH2-terminal Sequencing

Table 1 summarizes the amino-terminal sequence analysis of protein bands observed by reduced SDS-PAGE (Figure 2B) in pd-FIX, CHO-rhFIX and the biologically active pool (high acidic) tg-rhFIX obtained by CHT. These cleavages are consistent with those commonly attributable to that made by plasmin (12). The degraded species appearing within the therapeutic grade pd-FIX and CHO-rhFIX were detected at 56 kDa, 48 kDa, 40 kDa, 30 kDa, 18 kDa, and 8 kDa with amino terminal sequences corresponding to FIX zymogen, FIX γ , FIX α -HC, FIX $\alpha\gamma$ -HC, LC and the γ -peptide. The similar degraded species appeared in tg-rhFIX were 54 kDa, 46 kDa, 38-40 kDa, 30 kDa, 17-18 kDa and 8

kDa. In contrast to the more highly fractionated, therapeutic grade pd-hFIX and CHO-rhFIX preparations, only the tg-rhFIX pool contained the FIX $\alpha\beta$ -HC, which is the activated FIX, also FIXa, that possessed a MW of about 28-29 kDa.

Band # ¹	M. W. (kDa)	N-terminal a.a. Sequence	Predicted FIX Species	FIX Sample
1	56	YNSGK	FIX Zymogen	pd-FIX
2	48	YNSGK	FIX γ ²	
3	40	AETVF	FIX α -HC ³	
4	30	AETVF	FIX $\alpha\gamma$ -HC ⁴	
5	18	YNSGK	LC	
6	8	SALVL	γ -peptide	
7	56	YNSGK	FIX Zymogen	CHO-rhFIX
8	48	YNSGK	FIX γ	
9	40	AEAVF	FIX α -HC	
10	30	AETVF	FIX $\alpha\gamma$ -HC	
11	18	YNSGK	LC	
12	8	SALVL	γ -peptide	
13	54	YNSGK	FIX Zymogen	tg-rhFIX CHT high acidic eluate
14	46	YNSGK	FIX γ	
15	38-40	AETVF	FIX α -HC	
16	30	AETVF	FIX $\alpha\gamma$ -HC	
17	28-29	VVGGE	FIX $\alpha\beta$ -HC ⁵	
18	17-18	YNSGK	LC	
19	8	SALVL	γ -peptide	

Table 1. NH₂-terminal amino acids (a. a.) sequence of all bands on reduced SDS-PAGE of FIX samples and predicted FIX species. ¹The band number is according to the number shown on the reduced SDS PAGE of FIX samples (Figure 2B). ²The FIX γ is the FIX zymogen without the c-terminal γ -peptide ³The FIX α -HC is the FIX zymogen without the light chain (LC); it the heavy chain (HC) with activation peptide (AP) ⁴The FIX $\alpha\gamma$ -HC is the FIX α -HC without the c-terminal γ -peptide ⁵The FIX $\alpha\beta$ -HC is the heavy chain alone.

The FIX $\alpha\gamma$ -HC retained the activation peptide but had the γ -peptide removed and was therefore inactivated (12). In contrast, pd-FIXa consists of a FIX $\alpha\beta$ -HC having an intact protease domain where when paired with an intact light chain possesses a high specific activity of about 6000 U/mg (measured plasma derived human activated FIX). Zymogen forms of FIX typically has specific activity of about 150-200 U/mg. Thus, the high

specific activity of high acidic pool tg-rhFIX from CHT is seen to be caused by the presence of FIXa.

Separate Active Tg-rhFIX Zymogen from CHT Eluate by HPLC-SEC

The high acidic tg-FIX was loaded on the HPLC-SEC in the presence of calcium ion.

Three peaks of species with different biological activity were isolated (Figure 3A, Table 2). Pool 1 (Figure 3B, lane 2) exhibited both a low biological activity by coagulation assay and low extent of γ -carboxylation (Table 3). Pool 2 (Figure 3B, lane 3) and Pool 3 (Figure 3B, lane 4) showed both a high specific coagulation activity (Table 2) and high extent of γ -carboxylation (Table 3) that was closed to that obtained for pd-FIX and CHO-rhFIX. Both SEC pool 1 and 2 contained primarily FIX and FIX α and very minor amounts of FIX $\alpha\gamma$ but not activated FIX. Fraction 3 showed the highest specific activity of any FIX preparation which correlated well with a high content of FIXa and the respective FIX $\alpha\beta$ -HC and LC content identified by N-terminal sequencing. This confirmed the origin of the high overall activity seen in the starting CHT eluate.

Samples	Retention Time (min)	Specific activity (Units/mg ¹)
CHT Eluate	N/A	1681 \pm 187
SEC Pool 1	16.5-20	11 \pm 1
SEC Pool 2	20.5-22.5	155 \pm 13
SEC Pool 3	23-28	3203 \pm 890
Mononine	N/A	181 \pm 8
BeneFIX	N/A	178 \pm 14

Table 2. The tg-rhFIX CHT most acidic eluate SEC pools and their FIX specific activity.

¹One International Unit is the amount of factor IX activity present in 1 mL of pooled, normal human plasma. The specific activity of plasma derived human FIX is greater than or equal to 200 IU per milligram of protein.

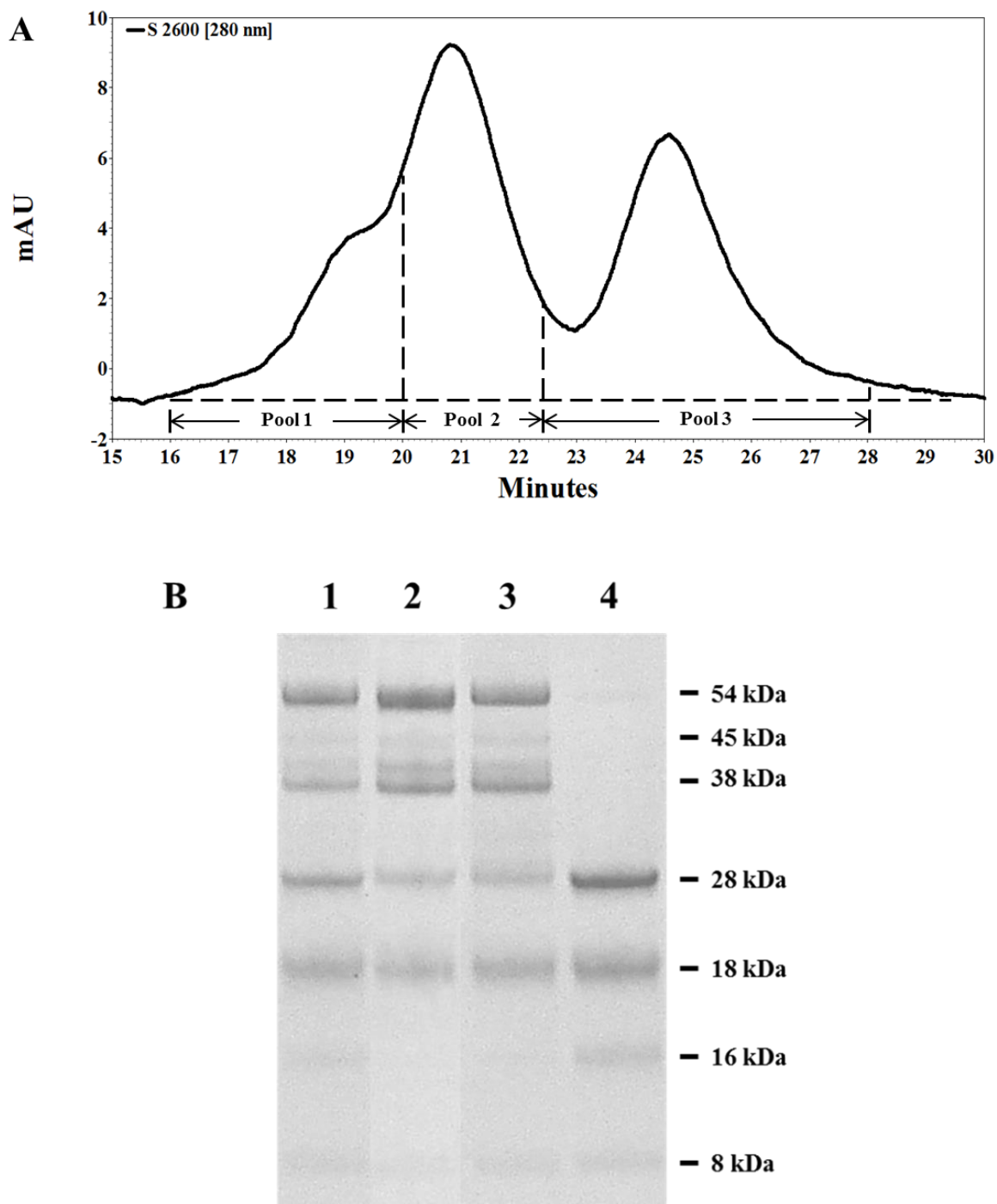


Figure 3. Fractionation by HPLC-SEC of high acidic tg-rhFIX population. Panel A. HPLC-SEC profile of most acidic tg-rhFIX pool. SEC Pool 1, 2, and 3 collected from HPLC-SEC are as indicated. Panel B. Reduced SDS-PAGE of SEC pools. Lane 1. Starting tg-rhFIX CHT eluate pool used for SEC fractionation; Lane 2, SEC pool 1; Lane 3, SEC pool 2; Lane 4, SEC pool 3. All samples loaded 2 ug.

Gla Extent	pd-FIX	CHO-rhFIX	SEC Pool	
			1	2
≥ 10	100%	100%	$< 20\%$	$\geq 80\%$
< 10	0	0	$\geq 80\%$	$< 20\%$

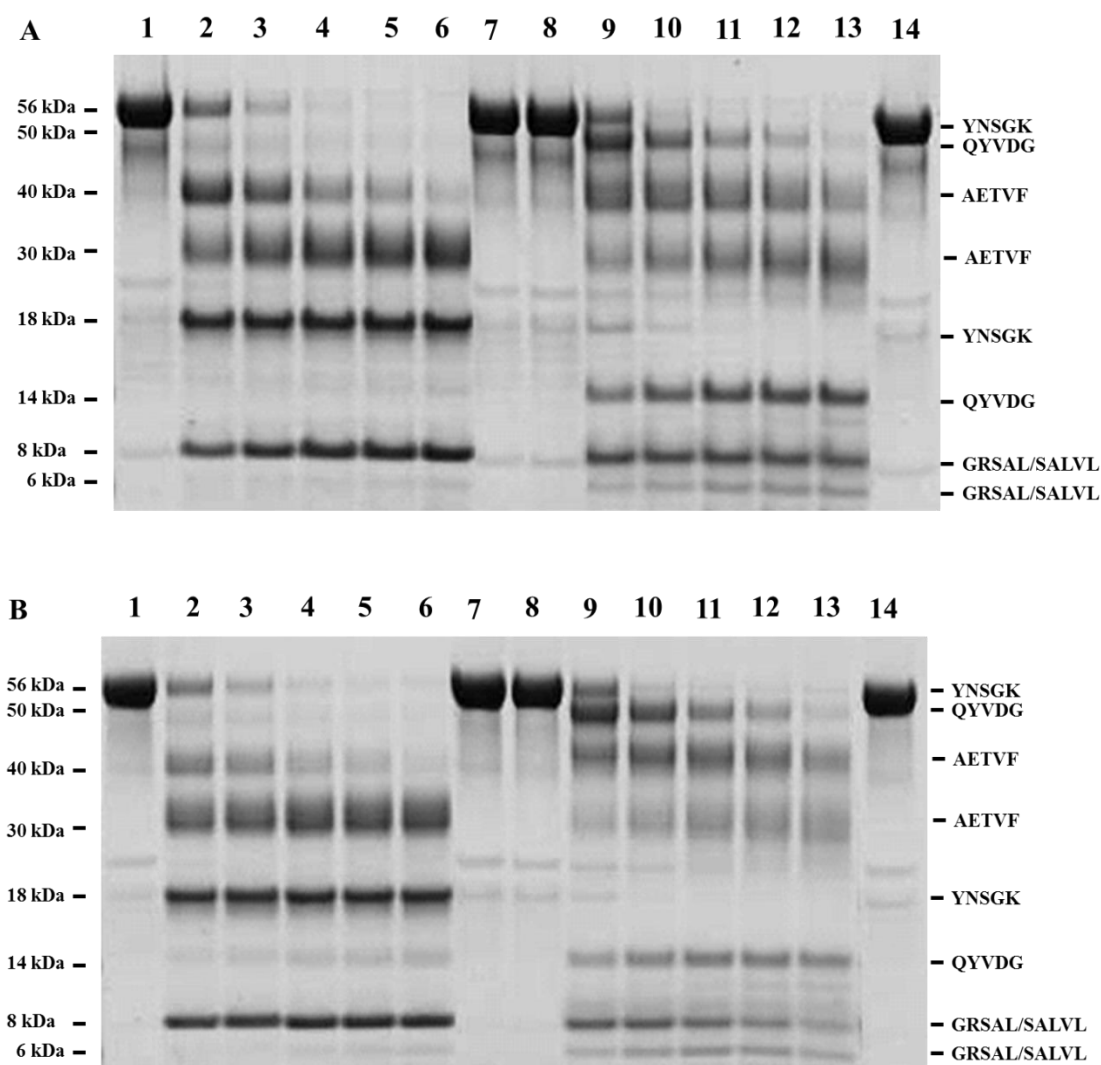
Table 3. Extent of γ -carboxyglutamic acid (Gla) of FIX samples.

***In Vitro* Plasmin Digestion of FIX Zymogen**

Time course plasmin digests of pd-FIX (Fig. 4A), CHO-rhFIX (Fig. 4B) and HPLC-SEC pool 1 (low Gla content) and 2 (high Gla content) of tg-rhFIX (Figures 4C and D) done with Ca^{2+} (lanes 2-6) or in a chelated environment (lanes 9-13) were evaluated by SDS-PAGE. In general, for all FIX species evaluated here, the pathway of proteolysis at α (Arg^{145}) and γ (Arg^{318}) sites by plasmin took place at a faster rate in the presence of calcium than in EDTA. The rate and pathway of proteolysis in the presence of Ca^{2+} for SEC pool 2 tg-rhFIX was similar to that of pd-FIX and CHO-rhFIX proceeding primarily to $\text{FIX}\alpha\gamma\text{-HC}$. This showed that the origin of $\text{FIX}\alpha\gamma\text{-HC}$ in the high acidic tg-rhFIX was likely caused by endogenous porcine plasmin in the milk. However, no evidence of the cleavage at Arg^{180} and production of FIXa by plasmin were observed.

The proteolysis at α and γ sites in a chelated environment was also similar for pd-FIX, CHO-rhFIX, tg-rhFIX SEC pool 1 and 2 (Fig. 4C and D, lane 9-13). In contrast to that in the presence of Ca^{2+} (Fig. 4C and D, lane 1-6), the proteolysis of the most carboxylated FIX species in 10 mM EDTA all shifted to a pathway that generated a cleavage in the light chain at Lys^{43} and releasing the Gla domain. While $\text{FIX}\alpha$ was still made, but at a

slower mode, same as the proteolysis at Arg³¹⁸. The proteolysis of LC at Lys⁴³ in less carboxylated tg-rhFIX, SEC pool 1, behaved similarly in either a Ca²⁺ or chelated environment. However, the proteolysis of the Arg¹⁸⁰ and Arg³¹⁸ in less carboxylated tg-rhFIX behaved same as the other three FIX samples in both environments. We show for the first time that a non-native conformation due to a low extent of γ -carboxylation results in a prominent presentation of Lys⁴³ site in the light chain that results in cleavage after. Our findings with the highly carboxylated SEC pool 2 tg-rhFIX are consistent with the previous observations of (22).



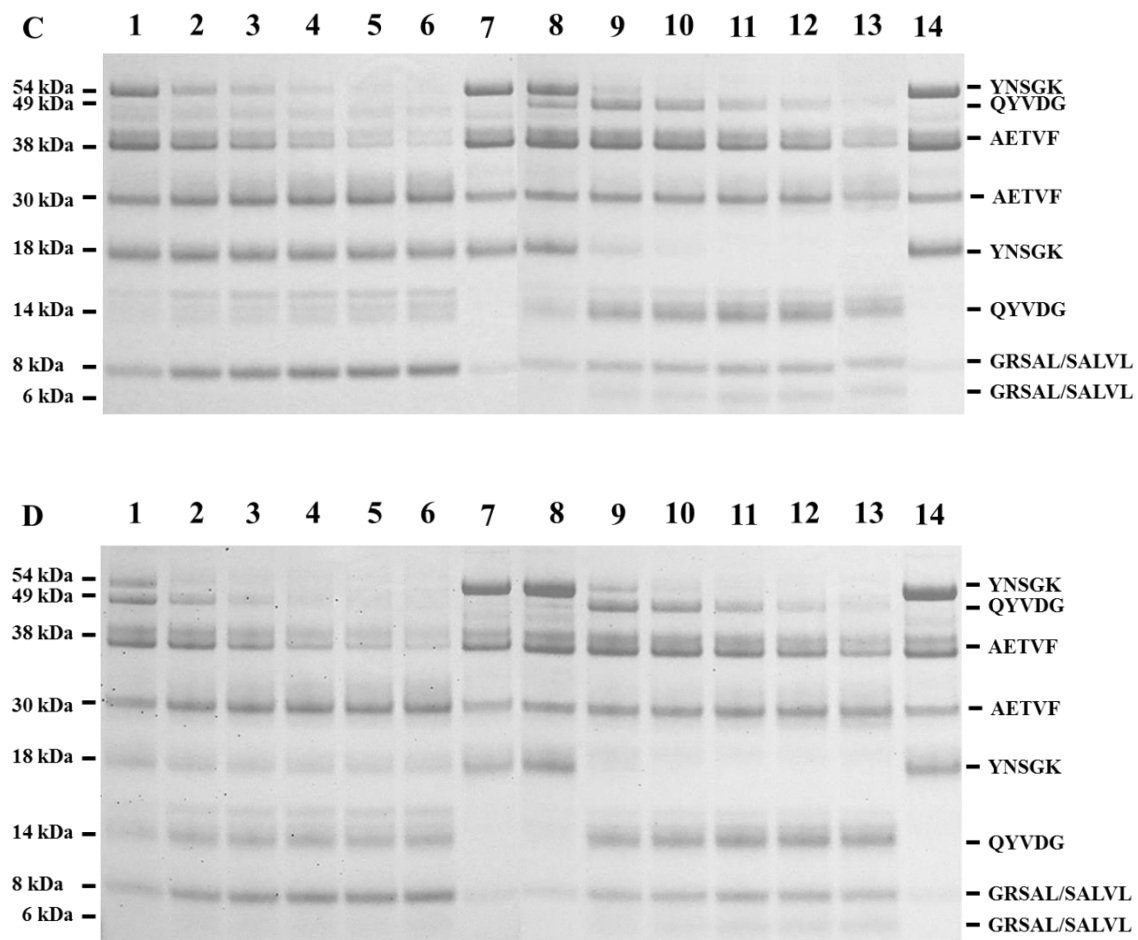


Figure 4. Reduced SDS-PAGE of time course plasmin digestion of FIX in Ca^{2+} and in chelated environments. Panel A. pd-FIX; Panel B. CHO-rhFIX; Panel C. SEC Pool 2 tg-rhFIX; Panel D SEC Pool 1 tg-rhFIX. For all panels: Lane 1-6. plasmin treated sample in 5 mM CaCl_2 at $t = 0, 10, 20, 40, 60,$ and 120 minutes, respectively; lane 7, untreated sample at $t = 120$ minutes. Lane 8-13 plasmin treated sample in 10 mM EDTA at $t = 0, 10, 20, 40, 60,$ and 120 minutes, respectively; No plasmin added sample in 10 mM EDTA at $t = 120$ minutes. All lanes have $3.75 \mu\text{g}$ of FIX sample loaded. NH₂-amino terminal sequencing of each protein species as indicated.

***In Vitro* Plasmin Digestion of Activated FIX**

We examined FIXa stability in the presence of plasmin. The pd-FIXa, and activated CHO-rhFIX and tg-rhFIX SEC pool 2 (activated by FXIa *in vitro*) all exhibited a similar resistance to proteolysis at Arg³¹⁸ by plasmin in the presence of Ca^{2+} (Figure 5).

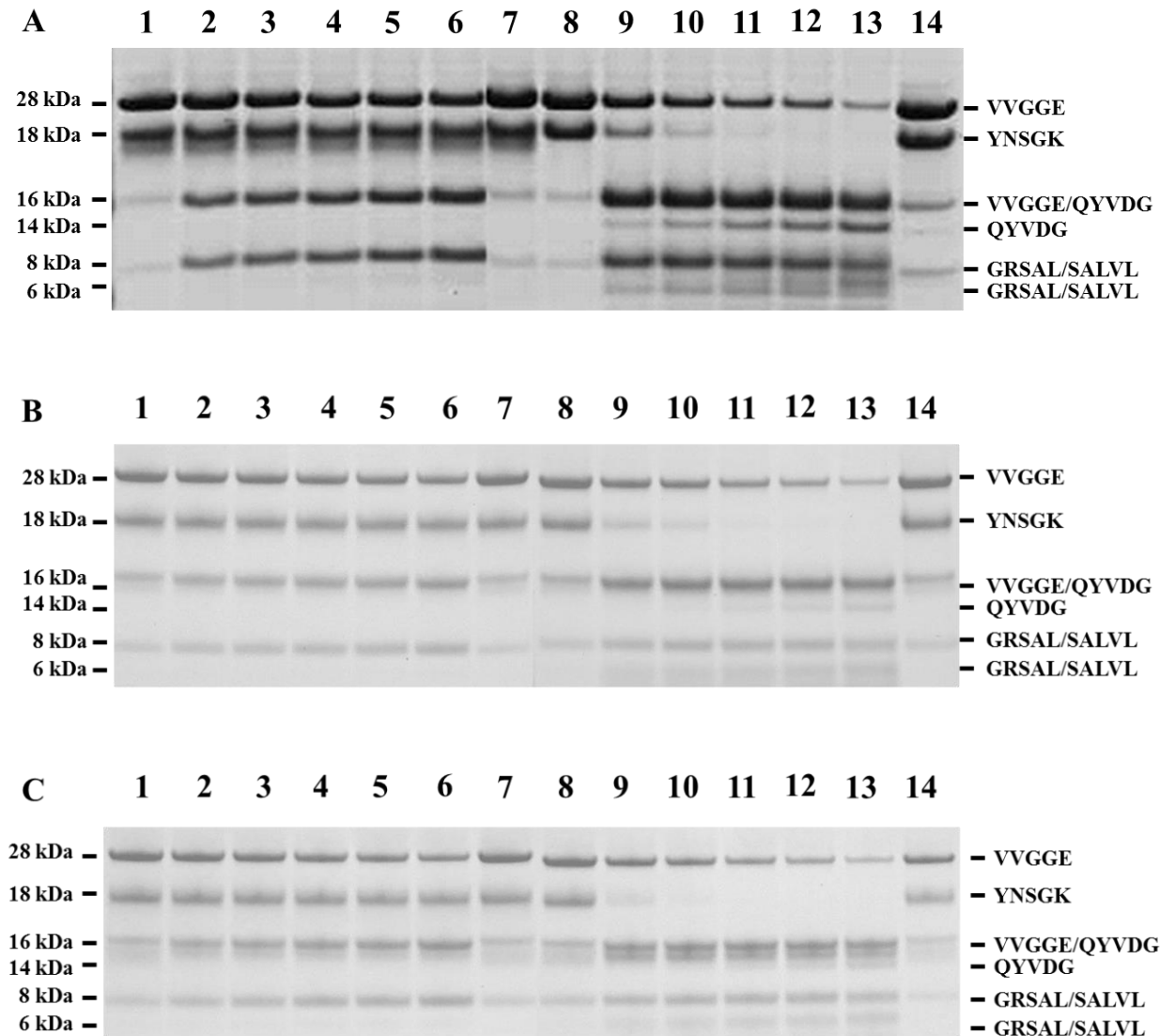


Figure 5. Reduced SDS-PAGE of time course plasmin digestion in Ca^{2+} and in chelated environments of activated FIX. Panel A. pd-FIXa; Panel B. CHO-rhFIXa; Panel C. Fraction 2 tg-rhFIXa; For all panels: Lane 1-6. plasmin treated sample in 5 mM CaCl_2 at $t = 0, 10, 20, 40, 60,$ and 120 minutes, respectively; lane 7, untreated sample at $t = 120$ minutes. Lane 8-13 plasmin treated sample in 10 mM EDTA at $t = 0, 10, 20, 40, 60,$ and 120 minutes respectively; Lane 9: no plasmin added sample in 10 mM EDTA at $t = 120$ minutes. All lanes have $3.75 \mu\text{g}$ of FIX sample loaded. NH₂ -amino terminal sequencing of each protein species as indicated.

The proteolytic product was FIX γ -HC. In a chelated environment, all FIXa species were rapidly proteolyzed at Lys⁴³. This pattern is opposite from the proteolysis of FIX zymogen, and the only difference between the substrates was the activation peptide.

CONCLUSIONS

Consistent with the previous findings for fully carboxylated pd-FIX, we observed that both highly carboxylated CHO-rhFIX and tg-rhFIX will undergo efficient plasmin cleavage at Arg¹⁴⁵ in the presence of calcium to form FIX α then FIX $\alpha\alpha$. But we did not see the cleavage at Arg¹⁸⁰ which form the activated FIX. Although formation of FIXa from tg-rhFIX zymogen in milk was not directly caused by plasmin, the formation of the intermediate of activated FIX promotes the formation of the final activated tg-FIX by other protease, e.g. trypsin, in the milk. The proteolysis at Lys⁴³ in light chain of FIX was noticed in the chelated environment, it was also seen in the low γ -carboxylated tg-rhFIX even in the presence of Ca²⁺. This indicated the Ca²⁺ induced conformational change of the Gla domain prevented it being proteolyzed by the plasmin. In order to decrease or eliminate the proteolysis of plasmin, specific protease inhibitor, e.g. ϵ -aminocaproic acid, and EDTA are required.

REFERENCE

1. Kaufman, R., Wasley, L., Furie, B., Furie, B., and Shoemaker, C. (1986) Expression, purification, and characterization of recombinant gamma-carboxylated factor IX synthesized in Chinese hamster ovary cells. *Journal of Biological Chemistry* **261**, 9622-9628
2. White 2nd, G., Beebe, A., and Nielsen, B. (1997) Recombinant factor IX. *Thrombosis and haemostasis* **78**, 261

3. Liebman, H. A., Furie, B. C., and Furie, B. (1987) The factor IX phospholipid-binding site is required for calcium-dependent activation of factor IX by factor XIa. *J Biol Chem* **262**, 7605-7612
4. Kirchhofer, D., Lipari, M. T., Moran, P., Eigenbrot, C., and Kelley, R. F. (2000) The tissue factor region that interacts with substrates factor IX and Factor X. *Biochemistry* **39**, 7380-7387
5. Blostein, M. D., Furie, B. C., Rajotte, I., and Furie, B. (2003) The Gla domain of factor IXa binds to factor VIIIa in the tenase complex. *J Biol Chem* **278**, 31297-31302
6. Aslam, M., and Hurley, W. (1998) Peptides generated from milk proteins in the bovine mammary gland during involution. *Journal of dairy science* **81**, 748-755
7. Kaminogawa, S. Y., K. . (1972) Decomposition of b-casein by milk protease:similarity of the decomposed products to temperature-sensitive abd R-caseins. *Agric. Biol. Chem.* 1972 **36**
8. Kaminogawa, S. M., H.; Yamauchi, K. . (1972) Comparison of bovine milk protease with plasmin. *Agric. Biol. Chem.* **36**
9. Reimerdes, E. H. (1983) New aspects of naturally occurring proteases in bovine milk. *Journal of dairy science* **66**, 1591-1600
10. Benfeldt, C. L., L. B.; Rasmussen, J. T.; Andreasen, P. A.; Petersen, T. E. . (1995) Isolation and characterization of plasminogen and plasmin from bovine milk. *Int. Dairy J.* **5**
11. Samis, J. A., Ramsey, G. D., Walker, J. B., Nesheim, M. E., and Giles, A. R. (2000) Proteolytic processing of human coagulation factor IX by plasmin. *Blood* **95**, 943-951
12. Samis, J. A., Ramsey, G. D., Walker, J. B., Nesheim, M. E., and Giles, A. R. (2000) Proteolytic processing of human coagulation factor IX by plasmin. *Blood* **95**, 943-951
13. Monroe, D. M., Noyes, C. M., Straight, D. L., Roberts, H. R., and Griffith, M. J. (1985) Activation of normal and abnormal human factor IX with trypsin. *Archives of biochemistry and biophysics* **238**, 490-496
14. Giles, A. R., Nesheim, M. E., Herring, S. W., Hoogendoorn, H., Stump, D. C., and Heldebrant, C. M. (1990) The fibrinolytic potential of the normal primate following the generation of thrombin in vivo. *Thromb Haemost* **63**, 476-481
15. Lindsay, M., Gil, G.-C., Cadiz, A., Velander, W. H., Zhang, C., and Van Cott, K. E. (2004) Purification of recombinant DNA-derived factor IX produced in transgenic pig milk and fractionation of active and inactive subpopulations. *Journal of Chromatography A* **1026**, 149-157
16. Clark, A. J., Bessos, H., Bishop, J. O., Brown, P., Harris, S., Lathe, R., Mcclenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B. A., and Wilmut, I. (1989) Expression of Human Anti-Hemophilic Factor-Ix in the Milk of Transgenic Sheep. *Bio-Technol* **7**, 487-492
17. Brunisholz, R. A., Lerch, P. G., Schaller, J., Rickli, E. E., Lergier, W., Manneberg, M., and Gillesen, D. (1981) Comparison of the primary structure of the N-terminal CNBr fragments of human, bovine and porcine plasminogen. *European journal of biochemistry / FEBS* **114**, 465-470

18. Brunisholz, R. A., and Rickli, E. E. (1981) Primary structure of porcine plasminogen. Isolation and characterization of CNBr-fragments and their alignment within the polypeptide chain. *European journal of biochemistry / FEBS* **119**, 15-22
19. Jagadeeswaran, P., and Sheehan, J. P. (1999) Analysis of blood coagulation in the zebrafish. *Blood Cells, Molecules, and Diseases* **25**, 239-249
20. Gil, G.-C., Velandar, W. H., and Van Cott, K. E. (2008) Analysis of the N-glycans of recombinant human Factor IX purified from transgenic pig milk. *Glycobiology* **18**, 526-539
21. Wolberg, A. S., Morris, D. P., and Stafford, D. W. (1997) Factor IX activation by factor XIa proceeds without release of a free intermediate. *Biochemistry* **36**, 4074-4079
22. Enfield, D. L., and Thompson, A. R. (1984) Cleavage and activation of human factor IX by serine proteases. *Blood* **64**, 821-831

CHAPTER 3

BIOENGINEERING OF THE MAMMARY GLAND OF PIG: INCREASED PRO-PEPTIDE PROCESSING OF FACTOR IX IN THE MILK OF TRANSGENIC PIGS BY CO-EXPRESSION OF FURIN

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ABSTRACT

28,008 patients worldwide had been diagnosed hemophilia B, which was congenital deficiency in coagulation factor IX (F9), and 1,005,358,368 IU F9 concentrates was consumed to treat those patients in 2012 (1). However, still about 70-80% patients received inadequate or no treatment because of unavailable and/or unaffordable factor concentrates (2). It is desired to manufacture safe and more economical F9. The higher cell density of the mammary gland, large quantity of milk production, and human-like post-translational modifications have been demonstrated to make bioactive recombinant (r) F9 at higher levels in mammary gland of transgenic pig than is currently done by using CHO cells. Cleavage of pro-peptide of nascent rF9, rate limit enzymatic modification, cannot be fully achieved by endogenous porcine furin at the high rF9 expression rate in transgenic mammary epithelium. Our data showed about 10-30% of whole rF9 population was inactive pro-peptide attached rF9 (pro-rF9). In this study, we constructed transgene with both the truncated human furin and F9 gene and generated the pig by somatic cell nuclear transfer (SCNT) technology. We demonstrate that recombinant (r) furin was co-expressed with rF9 more than 2000 U/ml milk, and it converted the inactive pro-rF9 into active rF9 both *in vivo* and *in vitro*. No evidence was shown the co-expressing rfurin interfered the over-express ($\geq 2\text{g/L}$ milk) and α -carboxylation of rF9.

INTRODUCTION

Hemophilia B is the congenital bleeding disorders and is caused by deficiency in functional coagulation factor IX (F9); by 2012, there were 28,008 patients worldwide (1,3). Prophylactic therapy, intravenous infusions of F9 per week, significantly reduces internal bleeding episodes (ie., into joints) and improves the quality of life (4,5). 1,005,358,368 IU F9 was consumed worldwide in 2012(1). However, about 70-80% patients, mostly in developing countries, received inadequate or no treatment because of the limited supplies and high costs of either recombinant(r) or pathogen screened, plasma-derived (pd-) F9 concentrates, whose average wholesale price is about US\$1.18 per IU(2,6). Of 15 licensed F9 concentrates, 14 are pdF9 which may result in prion and virus transmission risks(7); while only one is mammalian cell expression derived, which is recommended because of the safety reason (5). Thus, more economical and pathogen safe ways to manufacture rF9 are desired.

Like other Vitamin K dependent proteins of the coagulation cascade, F9 is a complex protein made by the liver while occurring in plasma at trace levels of only 5 µg/ml(8). The complex biosynthesis of F9 begins with a precursor polypeptide that then undergoes a number of post-translational modifications (PTMs): proteolytic cleavage of the propeptide, γ-carboxylation, β-hydroxylation, phosphorylation, and glycosylation(9). Mammalian cells, like Chinese hamster ovary (CHO) cells can make certain PTMs essential to biological activity in a comparable way to hepatocytes such as propeptide removal and γ-carboxylation(10-12). Unfortunately, these are still very low rates of synthesis that impede the use of current bioreactor technology by developing countries to produce rF9 for biotherapeutic use (6,13).

The economical rF9 production is also hindered by the low expression rate and low mammalian cell concentrations, about 10^6 cells/ml culture (6,14). To offset the restriction caused by low cell concentration in bioreactors, we and other groups have shown that the three orders of magnitude higher cell density of the mammary gland and demonstrated expressing bioactive rF9 at higher levels in pig milk than is currently done in the culture media of stainless steel bioreactors(6).

While the mammary gland is a prodigious vehicle for producing recombinant proteins at high concentrations, the combination of species specificity of the mammary gland to make certain PTMs while providing sufficient milk for production is still challenging. Transgenic animals, including mice and sheep, produced recombinant human F9 in their mammary glands, limited amount of bio-active F9 was secreted into the milk(14-16). Importantly, our previous work has shown that the pig has combined capability to make a diversity of PTMs which include glycosylation, a high volume of milk production (200-400 liters per year) (6,17,18) and is also multiparous. These features make it an efficient livestock in which to do further multiple gene, bioengineering by using somatic cell nuclear transfer (SCNT) technology(19). Examples of the PTM capabilities of the pig has been demonstrated by the expression of human F8 (20), protein C (21) , von Willebrand factor (VWF) (22) and F9 (9,23). In addition, the pig has the advantage as a specific pathogen free source of human biotherapeutics because it is not sensitive to prion exposure. Other livestock such as cows may need the *PRNP* gene required for the development of prion diseases to be knocked out to help ensure a prion free condition (24).

In order to fully take advantage of the high translational and secretory throughput of the mammary gland, higher PTMs processing rates must be made by multiple enzymes engineered into the transgenic livestock. In perturbing the expression of rF9 ten-fold from 0.2 g/l to greater than 2 g/l, about 10-30% rF9 had pro-peptide attached. The defective propeptide cleavage results in destabilizing the calcium-induced Gla domain conformation change and no pro-coagulation activity of pro-peptide attached FIX (25). Furin or paired basic amino acid cleaving enzyme (PACE), a Ca^{2+} dependent serine protease of the PC family, is involved in processing pro-peptides at dibasic sites with consensus sequence Arg-X-Lys/Arg-Arg (RXK/RR) (26). This includes the precursors of hormone: pro-nerve growth factor β (27), pro-insulin, pro-albumin, pro-complement C3(28,29), pro-von Willebrand factor(30), pro-factor IX(31) and protein C (32). Furin is localized primarily to the trans-Golgi network (TGN)(33-36), and also traffics between TGN and cell surface, and ER retention of furin blocked its activation resulting in no bioactivity (37,38). The order in location of carboxylase (ER) and furin (TGN) *in vivo* assure the pro-peptide removal after the carboxylation formed.

In terms of bioengineering of the mammary gland of a mouse, Drews et al. used furin under the control of the murine Whey Acidic Protein (mWAP) to increase the proteolytic processing of internal dipeptides of protein C(32). We here present the bioengineering of the pig mammary gland to provide sufficient furin activity to completely remove the pro-peptide and convert the inactive pro-rF9 to mature and active rF9 zymogen form at expression rate of 2 g/L.

METHODS

All buffer components were purchased from VWR International LLC (Radnor, PA) or Thermo Fisher scientific (Waltham, MA) or Sigma (St. Louis, MO) unless otherwise stated. All of the following solutions and media used in fetal fibroblast collection, transgenic cells producing, oocyte maturation, SCNT, and embryo reconstruction were filtered by using a 0.22 μm filter. All animal procedures were performed with an approved University of Missouri Animal Care and Use (ACUC) protocol. Unless stated otherwise, the following purifications were performed on a BioCAD Vision chromatography station (Applied Biosystems, Grand Island, NY). In order to minimize degradation, purification processes were performed at 4°C. The pd-F9 (Mononine, CSL Behring, King of Prussia, PA) and CHO-rhF9 (BeneF9, Pfizer, New York City, NY) used in the study were expired for clinical use however when used in experiments, exhibited full pro-coagulant activity. Both were kind gifts from James Brown (Lincoln, NE)

Fetal Fibroblast Collection

Landrace fetal fibroblasts cells (FFCs) were collected as described(39) with some modifications. Briefly, after removing the head, intestine, liver, limbs, and heart, the fetus was minced and digested individually in 20 mL of digestion media (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% (v/v) FBS, 200 units/ mL collagenase and 25 Kunitz/mL DNaseI) for 5 hrs at 38.5°C and 5% CO₂ in air. Digested cells were washed with DMEM, 15% fetal bovine serum (FBS) (Hyclone, Logan, UT) with 10 $\mu\text{g/mL}$ gentamicin, cultured overnight, then collected and frozen at -80 °C in aliquots in FBS with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

Production of gene constructs

The production of the WAP6FIX and WAP5Furin transgenes has been described in detail previously (32,40) and showed in Fig. 1A. F9 and furin constructs were linearized by *NotI*. After enzyme digestion, constructs were gel purified and stored at -20°C until transfection.

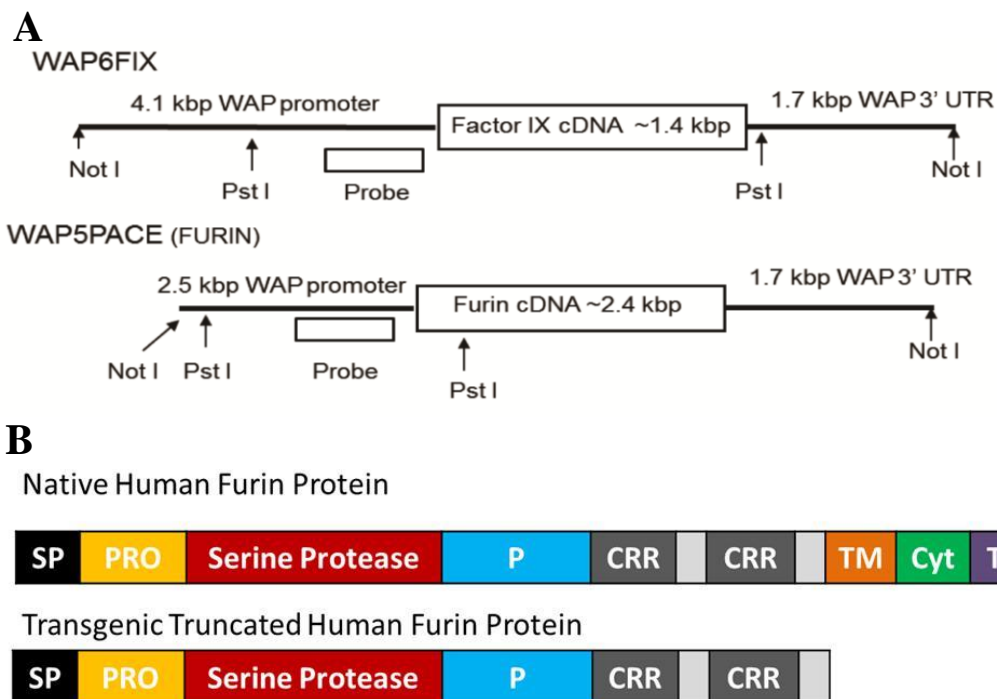


Fig.1. A. Transgene constructs schematic for WAP6FIX and WAP5Furin. The cDNA for human FIX and truncated human furin cDNA were placed under control of the 4.1 or 2.5 kbp mammary specific Whey Acidic Protein (WAP) promoters, respectively. **B. Domain schematic of the primary structure of both native and truncated human furin.** Domains indicated: Signal Peptide a.a.1-26 (SP); Propeptide a.a. 27-107 (PRO); Subtilisin-like Serine Protease domain a.a.132-447; activation maturation domain a.a.484-570 (P); Cystine-rich region (CRR); trans-membrane domain (TM); Cytoplasmic domain a.a. 759-762 (Cyt); Trans Golgi Network Signal domain (TGN).

Production of transgenic cells

Early passage number Landrace FFCs (1-2) were cultured in cell culture medium (DMEM supplemented with 15% (v/v) FBS, 2.5 ng/mL basic fibroblast growth factor (Sigma) and 10 µg/mL gentamicin.) overnight and grown to 75–85% confluency. Media was replaced 4 hours prior to transfection. FFCs were washed for 1–2 min with phosphate buffered saline (Invitrogen) and harvested with 0.05% trypsin-EDTA (Invitrogen; 1 mL per 75cm² flask). Cells were resuspended in cell culture medium, pelleted at 600 × g for 10 min, resuspended in 10 mL Opti-MEM (Invitrogen), and then quantified by using a hemocytometer and repelleted. Cells were resuspended in transfection media (75% cytosalts [120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄; pH 7.6, 5 mM MgCl₂] (van den Hoff et al. 1992) and 25% Opti-MEM (Gibco BRL Grand Island, NY)) and quantified again and the cell concentration was adjusted to 1×10⁶ cells/mL. Two hundred microliters cells were co-transfected by electroporation with linearize F9 (2µg), furin (2µg) constructs. Each electroporation utilized three consecutive 250-V, 1-ms square wave pulses administered through a BTX ECM 2001 (BTX, San Diego, CA) in 2 mm gap cuvettes. After electroporation, cells were plated in 100 mm dish at the concentration of 3000 cells per dish in cell culture medium. After 36 hours, cells were selected by the addition of geneticin (G418; 400 µg/mL) for 10-14 days until the formation of cell colonies. Genomic DNA from the cell colonies was used to verify the presence of all two genes by PCR. These cells then were stored in liquid nitrogen until used as donor cells for somatic cell nuclear transfer (SCNT).

Oocyte Maturation, SCNT, and Embryo Reconstruction

Fibroblast cells identified to have integration of both F9 and furin transgenes were used as donor cells for SCNT into enucleated oocytes followed by electrical fusion and activation (39). In brief, cumulus-oocyte cell complexes (COCs) were received in Phase I maturation medium from ART Inc. (Madison, WI) approximately 24 hours after harvest. COCs were then cultured in fresh Phase II maturation medium for 16 h. Maturation was in a humidified atmosphere of 5% CO₂ at 38.5°C. Expanded COCs were then vortexed in 0.1% hyaluronidase in Hepes-buffered Tyrode's medium containing 0.01% polyvinyl pyrrolidone for 4 min to remove the cumulus cells. Only oocytes having a visible first polar body (PB) with uniform cytoplasm were selected and placed in fresh manipulation medium (25 mM Hepes-buffered TCM199 with 3 mg/mL bovine serum albumin (BSA)) containing 7.5 µg/mL cytochalasin B overlaid with warm mineral oil. SCNT was conducted (39) by removing the PB, MII chromosomes and a small amount of surrounding cytoplasm of the oocyte by using a beveled glass pipette with an inner diameter of 17-20 µm. After enucleation, a donor cell was injected into the perivitelline space and placed adjacent to the recipient cytoplasm. The reconstructed embryos were fused and activated with 2 DC pulses (1 sec interval) of 1.2 kV/cm for 30 µsec provided by a BTX Electro-cell Manipulator 200 in fusion medium (0.3 M mannitol, 1.0 mM CaCl₂, 0.1 mM MgCl₂, and 0.5 mM Hepes, pH adjusted to 7.0–7.4). After fusion and activation, only the fused embryos were cultured into four well plates (Nunc, Denmark) containing 500 µL of PZM3 with 0.3% BSA and 500 nM Scriptaid at 38.5 °C and 5% CO₂ in humidified air for 14 to 16 hours, until embryo transfer(41).

Embryo Transfer and piglet production

More than 100 SCNT zygotes were surgically transferred to the oviducts of each surrogate on the day of, or one day after, the onset of estrus. Ultrasound was used to monitor the pregnancy. Piglets were delivered via cesarean section from surrogates by day 114-116 of gestation. Piglets are processed immediately and tissue samples were collected for establishment of cell lines and PCR genotyping. Piglets were then hand-raised until weaning (3-4 wks of age). After confirming genotype and reaching sexual maturity, the founders with both F9 and furin cDNA were mated to wild type pigs for production of animals for milking and phenotyping.

PCR Genotyping Assays

The integration of transgenes in the founders and offspring was conducted by using 10 ng of genomic DNA for each 25 μ L PCR reaction (Mastercycler Pro; Eppendorf, Hauppauge, NY). GoTaq polymerase (Promega, Madison, WI) was used as recommended by the manufacturers. PCR primers (Table 1) were used in under PCR cycling conditions of: 95°C for 3 minutes followed by 30 cycles at 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 45 seconds.

Gene	Primers	Fragment size
F9	Forward: 5'-TACCTCTTTGGCCGATTCAG-3'	430bp
	Reverse: 5'-GCTCCTTCATTTTCTCCGCT-3'	
Furin:	Forward: 5'- GCGAGAGGACCGCCTTTATCAAAGA-3'	293bp
	Reverse: 5'-TGGAAATCACCTGTGGCTGTCTTGC-3'	
eNOS	Forward: 5' -ACGAGCCTCCAGAACTCTTTGCTT-3'	243 bp
	Reverse: 5'- TTTCCAGCAGCATGTTGGACACTG-3'	

Table 1. PCR Genotyping primers and fragments size for transgenic pigs

Content and quality of genomic DNA loaded in the PCR reaction was confirmed by amplification of the endogenous swine eNOS gene using the following PCR conditions: 95°C for 3 minutes followed by 30 cycles at 95°C for 15 seconds, 59°C for 30 seconds, and 72°C for 45 seconds.

Pig milking

Transgenic pig milk was collected as previously described (42). Milk samples were collected twice daily on the following days (D5-10, then every three days until D35). Piglets were removed from the mother 30 minutes before milk sample collection and returned following sample collection. An injection of Oxytocin was given to the sow 10-15 minutes before sample collection.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were analyzed by SDS-PAGE stained with colloidal Blue gel stain (Invitrogen, Carlsbad, CA, USA) using Invitrogen 12% Bis-Tris Novex acrylamide precast gels and the Invitrogen Surelock XL apparatus (200 Volts for 1 hour). Briefly, samples were mixed with 4x LDS sample buffer (Invitrogen) and deionized water followed by heating at 74 °C for 10 min. For reduced gels, samples were mixed with 10x reducing agent (Invitrogen) prior to heating.

Western Blot

The gels were electro-blotted onto poly vinylidene fluoride (PVDF) membranes using a BioRad Transblot SemiDry Transfer Cell (Richmond, CA) for 30 min at 25 volts. For total F9, including all F9 species, blots were probed by rabbit polyclonal antibody (Pab) anti-F9 (F0652, Sigma-Aldrich St. Louis, MO); The furin was detected by using rabbit

Pab anti-furin (476-490) (F8806, Sigma-Aldrich St. Louis, MO); The mouse monoclonal antibody (Mab), PROFIX 3D2.6H12 (Green Mountain Antibody, Burlington, VT), was used in probing pro-peptide of F9. The secondary antibody (HRP-conjugated) anti- rabbit (A9169) and mouse (A4416) IgG (Sigma-Aldrich St. Louis, MO) was used to detect the primary antibody respectively. The chromogenic DAB substrate (Thermo, Rockford, IL) was used in color developing.

Quantification of Recombinant Human Factor IX (rF9) and Recombinant Human Pro-peptide attached Factor IX (pro-rF9) in Transgenic Milk

Western blot was performed as described before. Mononine or purified pro-rF9, as calibrator, was loaded along with the transgenic milk samples (triplicated). The concentration mononine or pro-rF9 was determined by measuring the optical density at 280 nm in a 1 cm quartz cuvette and using an extinction coefficient, $\epsilon^{1\%} = 13.4$. The pictures of the blots were analyzed by using the Image J(43,44). The amount of rF9 and pro-rF9 was then calculated by using the standard curve created by the calibrators.

Scaled-up Processing Pro-rF9 in Transgenic Milk by Incubating with Rfurin Co-expressed Transgenic Milk before Purification

Whole transgenic milk with rfurin co-expressed sample was first added to heparin sepharose fast flow (FF) (GE Healthcare Bio-Sciences AB, Sweden) resin at 1:1 (v/v) ratio and incubated for 30 min at 4 °C. The supernatant was collected (F9 depleted) and was then incubated with the whole transgenic milk with only rF9 expressed at 1:4 (v/v) ratio for 2 hour. All above milk samples were not treated with EDTA and any protease inhibitors.

Scaled-up Purification of High Acidic Recombinant Human Factor IX from Transgenic Swine Milk

High acidic rF9 was purified using the procedures adapted from previous study(23). The clarified transgenic milk samples was first diluted with the loading buffer (20 mM Tris, 50 mM NaCl, pH 7.4) at 1:3 ratio then loaded onto a heparin hyper D column, 26cm ID X 20 cm L (GE Healthcare Bio-Sciences AB, Sweden). The impurities were washed out by increasing [NaCl] to 200 mM, then the whole rF9 was eluted by increasing [NaCl] to 500 mM. The flow rate of the chromatography was 4 ml/min. The enriched rF9 sample was further loaded on the phenyl 650C column, 26 cm ID X 20cm L (Tosoh Bioscience, LLC) in the loading buffer (500 mM NaCl), the rF9 was collected in the flow through with flow rate at 4 ml/min. The rF9 sample was loaded on Q sepharose fast flow column (GE Healthcare Bio-Sciences AB, Sweden), 26 cm ID X 20cm L. The low acidic F9 population was washed out by increased [Ammonium acetate] to 537 mM, and the high acidic F9 was eluted at [Ammonium acetate] at 800 mM with the flow rate at 4 ml/min. All buffer exchanges of rF9 samples were performed by using the tangential flow filter (TFF) system (10 kDa MWCO, Pall Corporation, Port Washington, NY). All steps were performed at 4 °C.

Separate Active rF9 from High Acidic rF9 population by High Pressure Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC)

The high acidic rF9 was exchanged into SEC running buffer (20 mM Tris, 200 mM NaCl, 10 mM CaCl₂, pH 7.4) by using the Minimate tangential flow filtration systems with 10 kDa molecular weight cut off (MWCO) membrane (Pall Corporation, Port Washington, NY) or Amicon tube (10 kDa MWCO) (EMD Millipore, Billerica, MA). F9 sample was

loaded on a TSK gel G3000SW_{XL}, 7.8 mm ID x 30 cm, 5 μ m (particle size), equipped with a guard column, 6 mm ID x 4 cm, 7 μ m (particle size), (Tosoh Bioscience, LLC). The flow rate was 0.5 ml per min and duration of the run was 45 min with all major peaks eluting before 35 min. The chromatography were performed by using Knauer Smartline chromatography apparatus, including Manger 5000, Pump 1000 and UV Detector 2600 (Berlin, Germany), equipped with a Gilson FC 204 fraction collector (Middleton, WI). Both columns were equilibrated and run with 20 mM Tris base, 200 mM NaCl, 10 mM CaCl₂, pH 7.0. The fractions of each peak were pooled together for further analysis.

Purification of Pro-rF9 and rF9 from Transgenic Milk

Transgenic milk, no rfurin co-expressed with rF9 (K96 lactation day 24), was used to purify both rF9 and pro-rF9 subpopulation. The whole rF9 population (including both rF9 and pro-rF9 subpopulation) was purified by using the heparin hyper D column as described above. Then it was loaded on the immunoaffinity colum, mouse monoclonal antibody (Mab) anti-propeptide of pro-rF9(Green Mountain Antibody, Burlington, VT) immobilized Protein G Sepharose FF(GE Healthcare Bio-Sciences AB, Sweden) at 1 mg Mab/ml resin , 10 mm ID X 127 mm L (10 mL). The loading buffer was 20 Mm Tris, 50 mM NaCl, pH 7.4; the wash buffer was the loading buffer with 500 mM NaCl with the flow rate at 150 cm/hr. The rF9 subpopulation was achieved in the flow through. The pro-rF9 was eluted by using 0.1 M Glycine, pH 2.8 and then neutralized by 1:10 (v/v) 1 M Tris, pH 8.5 to the eluate.

Quantitation of total factor IX activity

Total F9 activity was determined using the activated partial thromboplastin time (aPTT) assay (45). Briefly, 50 μ l each of PTT Automate 5 reagent (Diagnostica Stago, Inc.,

Parsippany, NJ, USA), factor IX deficient plasma (George King Bio-Medical, Overland Park, Kansas, USA), and a sample of interest were added to a cuvette and incubated at 37 °C for 3 min. 50 µL of 25mM CaCl₂ (Sigma) was then added and the clotting time was measured using the STart Hemostasis Analyzer (Diagnostica Stago, Inc., Parsippany, NJ). The normal human plasma (Diagnostica Stago, Inc., Parsippany, NJ) was used as the standard, assigning 1 unit of F9 clotting activity per milliliter of plasma. The concentration of purified rF9 was determined by measuring the optical density at 280 nm in a 1 cm quartz cuvette and using an extinction coefficient, $\epsilon^{1\%} = 13.4$.

NH₂-terminal sequencing of the plasmin cleavage products of F9

Amino acid sequencing was performed at the University of Nebraska Medical Center, Protein Structure Core Facility. Protein samples were subjected to SDS-PAGE and transferred to PVDF as described above. The membrane was stained with Colloidal Blue and bands excised and subjected to Edman degradation using Applied Biosystems Procise protein sequencer, a Hitachi 8800 Amino Acid Analyzer and a Michrom MAGIC HPLC equipped with a Diode Array Detector.

Purification of Recombinant Truncated Human Furin (rfurin) from Transgenic Milk with Rfurin Co-expressed

Transgenic milk sample (R1014, lactation day 5-22 pool), having rfurin co-expressed, was first diluted with DEAE loading buffer, 20 mM HEPES, 50 mM NaCl, 2 mM CaCl₂, 0.1% (w/v) Brij-35, pH 7.5, at 1:4 (v/v) ratio and centrifuged at 12000 rpm, 4 °C for 20 min. The supernatant (clarified milk) was loaded on the DEAE fast flow column (GE Healthcare Bio-Sciences AB, Sweden), 10 mm ID X 127 mm L (10 ml) with the flow rate at 150 cm/hr; and the furin fraction was eluted by increasing [NaCl] to 140 mM. The

DEAE eluate was exchanged to the CHT binding buffer (20 mM HEPES, 2 mM Na₂HPO₄, 0.1% (w/v) Brij-35, pH 7.5) and loaded on the Ceramic Hydroxyapatite, CHT (Bio-Rad, Hercules, CA) column, 10 mm ID X 127 mm L (10 ml) at 150 cm/hr. The flow through, which had rfurin, was collected. Rfurin fractions were exchanged to the Q Sepharose binding buffer (20 mM HEPES, 50 mM NaCl, 2 mM CaCl₂, 0.1% (w/v) Brij-35, pH 7.2) and loaded onto the Q Sepharose FF (GE Healthcare, Bio-Sciences AB, Sweden) column, 10 mm ID X 127 mm L (10 ml) with the flow rate at 150 cm/hr; the furin fraction was eluted at 140 mM NaCl. Final sample was exchanged to 20 mM HEPES, 2 mM CaCl₂, 0.1% (w/v) Brij-35, pH 7.5.

Quantification of Activity of Furin

Activity of Furin in Milk—Fluorometric assays were carried out essentially as described (46). Whole milk sample was diluted 1:50 to 1:200 with assay buffer (20 mM HEPES, 2 mM CaCl₂, 0.1% (w/v) Brij-35, pH 7.5). Calibrator is the 7- amino, 4-Methyl Coumarin (AMC), concentration is from 0.39 uM to 25 uM in assay buffer. 50 uL 100 uM substrate, L-PyroGlu-Arg-Thr-Lys-Arg-AMC (pERTKR-AMC) (R&D systems, Minneapolis, MN, USA) was incubated with the 50 uL diluted sample at room temperature. Fluorescence was measured by Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA) at λ_{ex} 342 nm; λ_{em} 440 nm with kinetic mode for up to 20 mins. The control consisted of substrate and assay buffer. 1 Unit of furin is equal to 1 pmol AMC/min in the above conditions.

Cleavage of Pro-peptide of Pro-rF9 by Partial Purified Recombinant Truncated

Human furin (rfurin) *in vitro*

Add 346 μL rfurin sample (43.35 U/mL), total activity was 15 U (cleaved 15 pmol prpeptides/min), to 200 μg (0.2 mg/mL, total 3500 pmol pro-rF9) purified pro-rF9 described above in furin activity assay buffer (20 mM HEPES, 2 mM CaCl_2 , 0.1% (w/v) Brij-35, pH 7.5). At each time point (0, 30min, 1, 2, 4, 8 and 24 hr), reaction was quenched by taking 20 μL sample and mixed with 4 X SDS-PAGE sample buffer (with or without 10 X reduced agent) for non-reduced, reduced SDS-PAGE respectively. Additional 50 μL sample was quenched by 50 μL 20 mM sodium citrate, pH 7.4 for the F9 activity (aPTT) assay. The reaction was in room temperature (25 $^{\circ}\text{C}$)

RESULTS

Generation F9 and Furin Co-expressed Pigs

Thirteen live female *F9* transgenic piglets were born from 5 litters. Six of 13 pigs were positive for *F9* and *FURIN* transgenes. Fifteen male *F9* transgenic piglets were born from 3 litters and all of them were positive for F9 and furin transgenes (Table 2).

Production of Female Transgenic Offspring for Milk Collection and Mendelian

Inheritance of the Transgenes

Male transgenic founder lines, Line A (78-2, 78-4), Line B (79-2) and Line C (80-4) were mated with wild type Landrace sows to produce the female offspring for milk production to test the foreign protein expression. Sixty-four offspring were produced from 5 litters. Of those 64 offspring, 26 were positive for *F9* and *FURIN* (Table 3, Figure 2). The

percentage of transgenic positive offspring is 41% which is closed to the expected ratio of 50% for Mendelian inheritance of the transgenic loci. Transgenic males were mated to a wild-type boar and allowed to farrow. Milk samples were collected from all three integration sites (Line A, B and C).

Litter	Litter size	Sex	Transgenic	Wild type
65	1	Female	0	1
66	1	Female	0	1
67	6	Female	4	2
68	4	Female	1	3
69	1	Female	1	0
78	6	Male	6	0
79	5	Male	5	0
80	4	Male	4	0

Table 2. Summary of production of rF9 transgenic founder pigs

Founder	Litter number	offspring	F9 and Furin	Wild type
78-2	122	16	6	10
78-4	123	15	1	14
79-2	125	10	4	6
80-4	23, 129	23	15	8
Total	5	64	26(41%)	38 (59%)

Table 3. Male founders mated to the wild type sows for production of female transgenic offspring

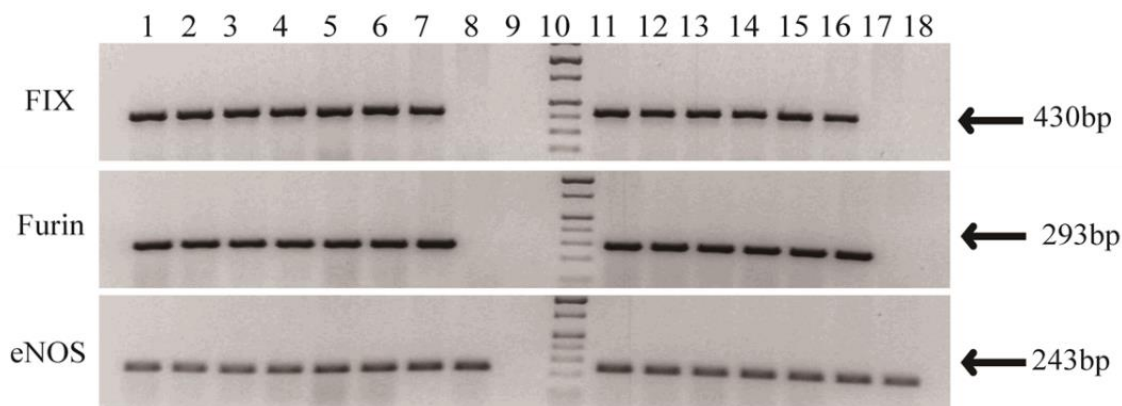


Fig. 2. Genotyping of F9 and Furin in the piglets of litter 78 and 79 by PCR. Eleven piglets from the two litters were carrying both transgenes; eNOS was used as endogenous gene control. Lane 1- 6, 6 piglets of litter 78; lane 7, positive control; lane 8, wild type genomic DNA; lane 9, negative control; lane 10, ladder; lane 11-15, 5 piglets of litter 79; lane 16, positive control; lane 17, wild type DNA; lane 18, negative control.

Limited Processing of the Pro-peptide of rF9 by Endogenous Porcine Furin in

Mammary Epithelium

The total rF9 expression rate was high in the transgenic milk, from 1.50 ± 0.02 g/L (Avg. \pm SD, n=3) to 2.30 ± 0.05 g/L (Avg. \pm SD, n=3) (Table 4). Western blot developed by antibody anti pro-peptide of human factor IX confirmed the existing of pro-rF9, which was not fully processed by endogenous porcine furin, in the transgenic milk (Fig. 3); and there was 9% to 27% pro-rF9 of total rF9 in the transgenic milk sample (Fig. 3, Table 4). The molecular weight of purified pro-rF9 was 57 kDa, 2 kDa more than the rF9 (Fig. 5A, lane 1). N-terminal amino acid sequencing showed the result, Thr-Val-Phe-Leu-Asp, matching the sequence of the pro-peptide of pro-rF9.

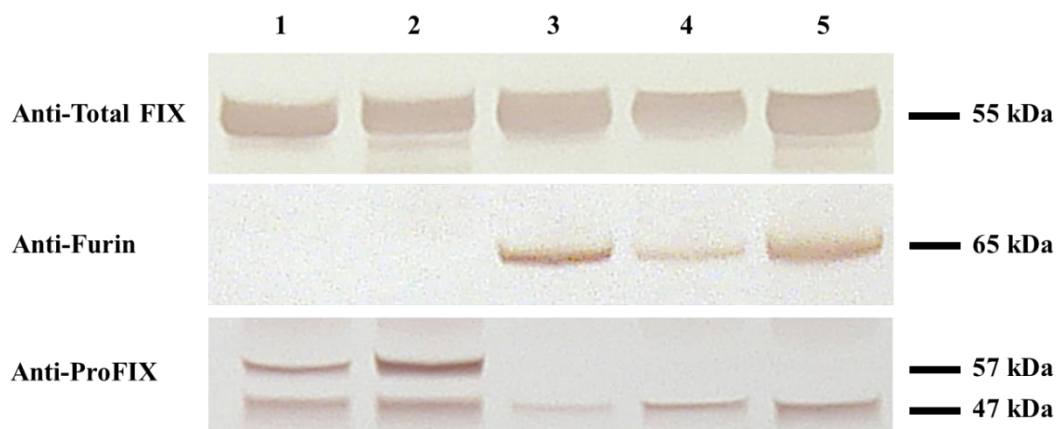


Fig.3. Western blot of milk samples. Milk samples included rF9 only expressed transgenic milk (K75 lactation day 11-23 pool and K101, lactation day 12-29 pool) and the rfurin co-expressed transgenic milk (R175 & 179 lactation day 5-35 pool, R180 lactation day 5-35 pool and R1014 lactation day 5-22 pool). All milk samples were diluted with 200 mM EDTA 1: 1 and skimmed as described in methods. Lane 1: K75 lactation day 11-23 pool; Lane 2: K101, lactation day 12-29 pool; Lane 3: R175 & 179 lactation day 5-35 pool; Lane 4: R180 lactation day 5-35 pool; and lane 5: R1014 lactation day 5-22 pool. 2 μ L of each milk sample was loaded on each lane. The 55 kDa bands indicated the F9 (including the rF9 zymogen and the pro-rF9); the 65 kDa bands indicated the rfurin; and the 57 kDa bands indicated the pro-rF9 (the 47 kDa bands were unspecific binding)

No F9 activity was detected for the pro-rF9 (Table 4). No furin antigen was detected by the Western blot developed using antibody anti-human furin; neither furin activity was detected in the milk sample.

PIG ID	Lactation Pool	[Total rF9] (mg/ml)	[Pro-rF9] (mg/ml) (Percentage of Pro-rF9 %)	Furin Activity (U/ml)
R175 & 179	Day 5-35	2.23 \pm 0.07		1402 \pm 40
R180	Day 5-35	1.60 \pm 0.35	Not Detected	539 \pm 217
R1014	Day 5-22	2.22 \pm 0.06		2214 \pm 33
K75	Day 11-23	2.30 \pm 0.05	0.21 \pm 0.05 (9%)	
K96	Day 10-25	1.63 \pm 0.36	0.25 \pm 0.04 (15%)	Not Detected
K101	Day 12-29	1.50 \pm 0.02	0.40 \pm 0.10 (27%)	

Table 4. Amount of total rF9, pro-F9 and rfurin in transgenic milk samples (n=3).

Rfurin Expressing and Processing Pro-peptide of rF9 *in vivo*

Not like the endogenous human furin, this truncated rfurin is eliminated transmembrane domain, cytoplasmic domain and trans-Golgi network (TGN) signal domain (Fig. 1B). This rfurin secretes into the milk, remaining its furin activity, while endogenous furin cycled in the TGN of epithelium. The Western blot developed by antibody anti-furin showed the rfurin signal, whose molecular weight is about 65 kDa (Fig. 5, lane F). And the furin activity reached up to 2214 ± 33 U/mL (Avg. \pm SD, n=3) in transgenic milk (R1014, lactation day 5-22 Pool). The expression of rfurin was stable during the lactation, and the more furin activity was detected during the late lactation (Fig. 4).

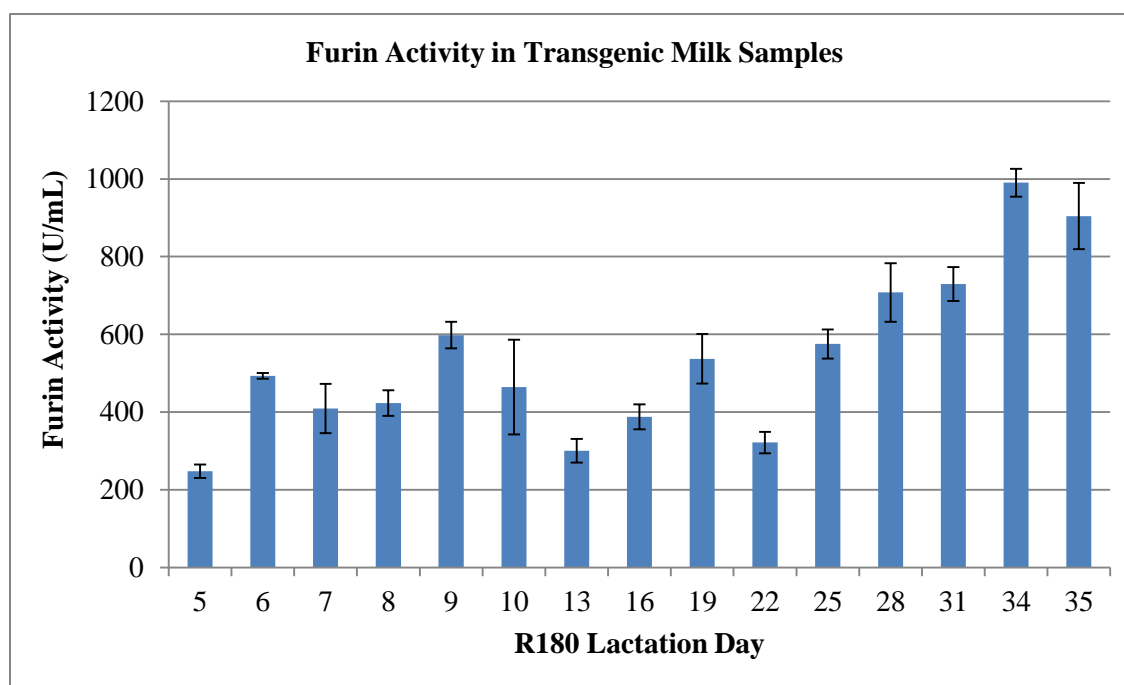


Fig. 4. Furin activity in transgenic milk. The furin activity of R180 transgenic milk samples (each lactation day) was measured. 1 Unit of furin is equal to 1 pmol AMC/min in the above conditions. The error bars is the standard deviation (n=3)

The co-expression of rfurin did not interfere the producing rF9 in transgenic milk, which reached 2.23 ± 0.07 g/L (Avg. \pm SD, n=3) (Table 4). No more pro-rF9 was detected (Fig. 3, Table 4).

Rfurin Processing Pro-rF9 *in vitro*

Rfurin was enriched (partially purified) from the transgenic milk sample. ~ 40% furin activity was recovered after 4 purification steps (Table 5). N-terminal amino acid sequencing of the 65kDa band (on the non-reduced SDS-PAGE) (Fig. 5A, lane F), Asp-Val-Try-Gln-Glu, matched the mature form of human furin (47). Purified pro-rF9 was incubated with partially purified rfurin at room temperature for up to 24 hours. We noticed the molecular weight of pro-rF9 shifted about 2 kDa after incubated with rfurin on both SDS-PAGE and western (Fig. 5A). Pro-rF9 signal was decreasing, while signal on total rF9 (including both pro-rF9 and rF9) remained same on the non-reduced western blots (Fig. 4B). Also, we noticed that the F9 specific activity of the sample increased after incubated with rfurin (Fig.5C).

Sample	Volume (ml)	Activity (U/ml)	Total Activity (U)	Activity Recovery %
Whole Milk	10	1925	19247	100
Clarified Milk	45	323	14527	75
DEAE Elution	85	142	12040	63
CHT Flow Through	175	50	8666	45
Q Elution	135	53	7209	37

Table 5. Purification table of isolation rfurin from transgenic milk (R1014 lactation day 5-22 pool)

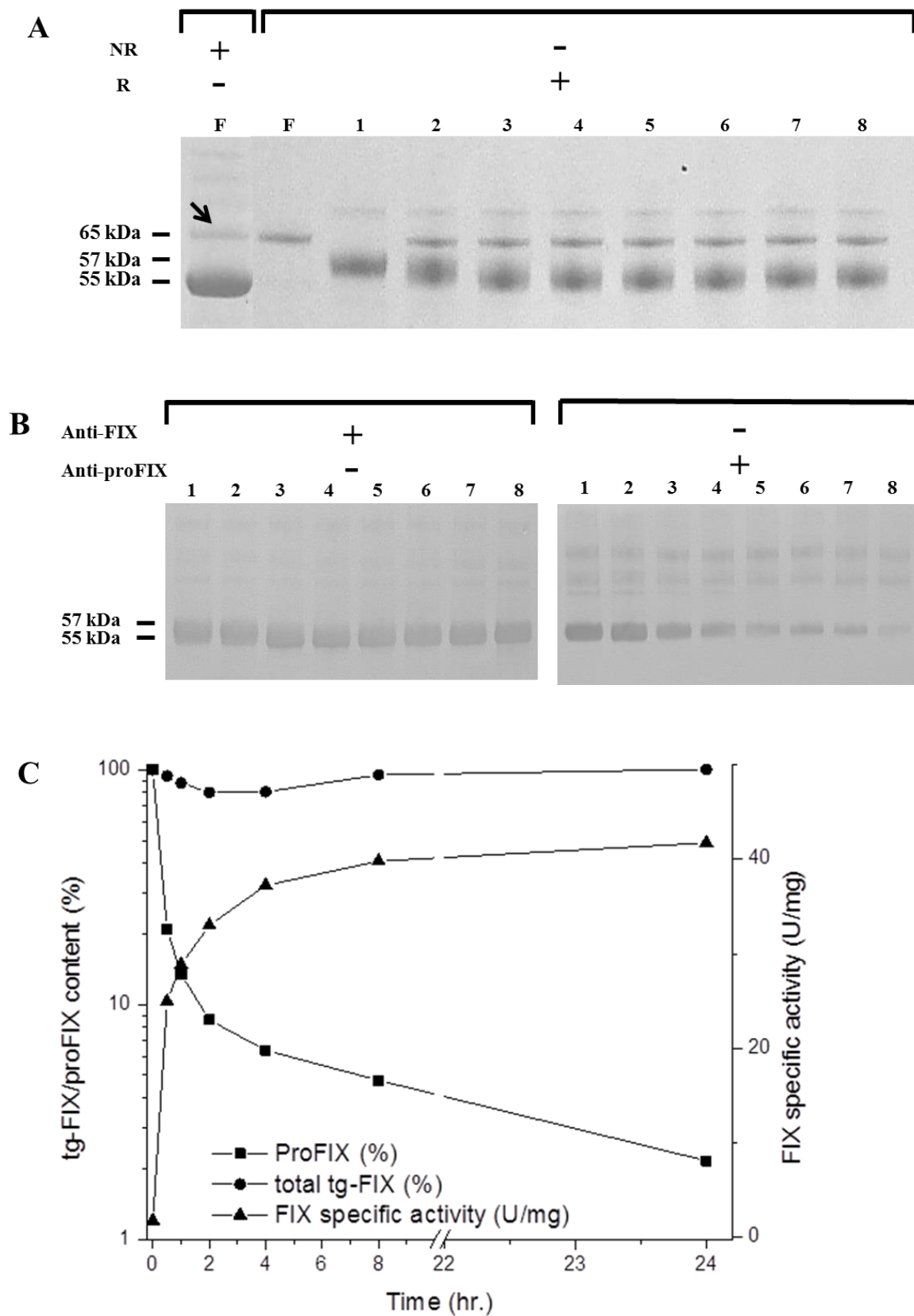


Fig. 5. RFurin cleave pro-peptide of pro-rF9 *in vitro*. **A.** Non-reduced and Reduced SDS-PAGE. **B.** Non-reduced western blot anti-FIX and proFIX. On non-reduced gel, lane F is partially purified rfurin sample and arrow pointing rfurin (65 kDa); On reduced gel, lane F is partially purified rfurin sample which loaded as same amount as in the reaction. The 65 kDa band is the band (~55 kDa) on non-reduced gel, not the rfurin. Lane 1: Purified pro-rF9 (57 kDa), same amount as the reaction loaded on the following lanes. Lane 2-8: Furin processing pro-rF9 *in vitro* reaction time= 0, 30 min, 1, 2, 4, 8 and 24 hr. **C.** Specific activity of pro-rF9; pro-rF9 and total rF9 content in the rfurin processing pro-rF9 reaction sample.

Scaled-up Purification of Active rF9 from Transgenic Milk

Both transgenic without rfurin co-expressed (K82, K89, K92 and K102 Pool) and with rfurin co-expressed (R185 Pool) were used to purify active rF9. The pro-rF9 in the transgenic milk was processed by incubated with rF9 depleted transgenic milk with rfurin co-expressed. The yield of active rF9 from the non- and rfurin co-expressed transgenic milk were 31.6 mg (active rF9) out of 1479 mg (total rF9) and 22.9 mg out of 1409 mg respectively; And there was no significant difference between the F9 specific activity of both active rF9 (Table 6).

Transgenic Milk	Heparin Eluate (mg)	Acidic Eluate (mg) / Recovery %	Active rF9 (mg) / Recovery %	Specific Activity (U/mg)
No rfurin co-expressed	1479	119 / 8 %	31.6 / 2 %	226 ± 42
Rfurin co-expressed	1409	90 / 6 %	22.9 / 2 %	216 ± 14

Table 6. Comparison of rF9 from both transgenic without rfurin co-expressed (K82, K89, K92 and K102 Pool) and with rfurin co-expressed (R185 Pool) milk by scaled-up purification.

Discussion

The demand of producing F9 proteins in a safe and highly efficient manner has increased. Based on the data from the World Federation of Hemophilia (Montreal, Canada), the consumption of F9 was approximately 250,100,000 U in the United States and

1,005,358,368 U worldwide in 2012(1). And we also need to consider the about 80% patients in developing and undeveloped area could not afford the high cost of the F9 concentrates. So the need for F9 concentrates is way more than the current consumption (3,48). Besides the high cost of hemophilia treatment, proteins derived from human plasma carry with them the risk of transmission of blood-borne diseases and infections(3). Considering bio-safety reasons, the application of recombinant human F9 products is widely recommended(49). A transgenic animal bioreactor is an attractive system for meeting the need for therapeutic recombinant proteins. Compared with other recombinant protein expression systems, transgenic animals have the ability to produce the biological active proteins approximately a 10-fold higher concentration of the target protein(6). In addition, a transgenic farm with a single purification facility should not cost more than US\$80 million(50). However, building a large-scale (10,000 liter bioreactor) manufacturing facility for a mammalian cell expression system is time consuming and costs US\$ 250–500 million(50). One successful example is ATryn, an anticoagulant, the first ever biologic product produced by a genetically engineered (GE) animal – a goat(51), which was approved by FDA to be used for the prevention of blood clots in patients in 2009.

The recombinant human F9 was expressed in several mammary gland of transgenic animals by using the SCNT technology. Very low amounts of inactive F9 have been detected in the transgenic sheep milk (52) . Zhang et al reported that the highest production of F9 in goat milk was 13.7 ng/mL 3 days after transfection plasmid DNA directly into the mammary gland lobule of a lactating goat and > 90% rF9 protein in milk appeared to be a gamma-glycosylated and biologically active (53). In mice, up to 60

mg/L at 50% of biologically active F9 have been reported (54). In contrast to the species listed above, Van Cott et al reported that full specific activity rF9 produced by porcine mammary gland cells of F9 was only 200 mg/L in the milk(55). Lindsay et al made pigs capable of producing rF9 at 2-3 g/L (23). However, with the 10-fold increasing in expression rate, the active rF9 decreased to only 10-20%, in addition, we noticed about 10-30% rF9 had pro-peptide attached, which was no pro-coagulation activity. The low percentage of active rF9 in total over-expressed rF9 is because of the rate limited enzymatic PTMs after nascent rF9 synthesis in mammary epithelia. To increase the yield of active rF9 by converting no-active pro-rF9 into active rF9 zymogen, we bioengineered the pig mammary gland by introducing truncated human furin gene to process the excessive propeptide. Three male founder lines, Line A (78-2, 78-4), Line B (79-2) and Line C (80-4) were mated with wild type landrace sows to produce the female offspring for milk production. Sixty four offspring were obtained in 5 litters (Table 3). Among these piglets, 26/64 (41%) expressed both rF9 and rfurin. Offspring of were either carrying two genes or totally wild type (Table 3) which implies linkage, and that the multiple genes might be integrated as clusters. The highest expression level of rF9 was 2.2 mg/mL milk in pig (R1014), 76-fold higher than production cell line which was 30 μ g/mL(56); and the furin activity in the same milk was more than 2000 U/mL. No significant difference in rF9 level compared to the pig (K75) without rfurin co-expressed. This indicates that the co-expressing rfurin did not suppress the rF9 expression. In contrast to the milk without rfurin co-expressed, no pro-rF9 was detected in the milk with rfurin co-expressed (Table 3). Unlike the endogenous furin, the co-expressed truncated rfurin was no transmembrane and following domain (Fig. 1B), so this rfurin secreted out

the cell rather than the endogenous furin cycled in the trans-Golgi network (TGN). This secreted rfurin not only processing nascent rF9 in cell but cleaving pro-peptide in the milk as well (Fig. 5). Because the rfurin does not have the transmembrane, which anchors the furin on the TGN membrane, we worried the over expressed rfurin moved back into the ER removing propeptide, which required by the carboxylase, before or during the γ -carboxylglutamic acids synthesis, which is required for F9 activity. The active rF9 was purified from both milk samples, with and without rfurin co-expressed, by using the same purification procedures (except the pro-peptide processing). The overall yield of active rF9 was similar, which indicated the co-expressed rfurin not interfering the carboxylation of glutamic acids. We also noticed in the 10-20% active rF9 subpopulation, almost half was activated rF9 or degraded rF9, which was active as rF9 zymogen before activation and/or degradation. This was partially caused by protease in milk activating and degraded rF9 during processing pro-rF9 *in vitro*. With the co-expressed the rfurin, the *in vitro* processing pro-rF9 was eliminated, and milk could be treated with protease inhibitors (i.e. ϵ -aminocaproic acid and benzamidine). The yield of active rF9 could further increase by decreasing the activation and degradation of rF9 during the processing.

In summary, we increased the quality of rF9 by co-expressed rfurin removing the pro-peptide of pro-rF9. rF9 was expressed at a level of 2.2 g/L of milk, and no pro-rF9 was detected in the milk. The consumption of rF9 in the U.S. can be estimated at 1.25 kg/year (250,100,000 U); and world consumption, at 5 kg/year(1). At 2 g/L rF9 expression rate and 400 L milk year⁻¹ pig⁻¹, and the yield of purified active rF9 zymogen, about 70 transgenic pigs could meet US's market and about 270 transgenic pigs can produce enough rF9 for the entire world.

Acknowledgement

This research was supported by the National Institutes of Health, National Heart, Lung and Blood Institute (R01 HL078944: cGMP Recombinant F9 for IV and Oral Hemophilia B Therapy

Thanks to Dr. Nick Masiello (rEVO Biologics) generous help in purifying recombinant human F9 from transgenic milk.

Reference

1. WFH. (2013) WFH Report on the Annual Global Survey 2012 Available at <http://www1.wfh.org/publications/files/pdf-1574.pdf>
2. Stonebraker, J. S., Bolton-Maggs, P. H. B., Brooker, M., Farrugia, A., and Srivastava, A. (2011) A study of reported factor IX use around the world. *Haemophilia* **17**, 446-455
3. Gater, A., Thomson, T. A., and Strandberg-Larsen, M. (2011) Haemophilia B: impact on patients and economic burden of disease. *Thromb Haemost* **106**, 398-404
4. Panicker, J., Warriar, I., Thomas, R., and Lusher, J. M. (2003) The overall effectiveness of prophylaxis in severe haemophilia. *Haemophilia : the official journal of the World Federation of Hemophilia* **9**, 272-278
5. Franchini, M., Frattini, F., Crestani, S., Sissa, C., and Bonfanti, C. (2013) Treatment of hemophilia B: focus on recombinant factor IX. *Biologics : targets & therapy* **7**, 33-38
6. Van Cott, K. E., Monahan, P. E., Nichols, T. C., and Velander, W. H. (2004) Haemophilic factors produced by transgenic livestock: abundance that can enable alternative therapies worldwide. *Haemophilia* **10 Suppl 4**, 70-76
7. Ludlam, C. A., Powderly, W. G., Bozzette, S., Diamond, M., Koerper, M. A., Kulkarni, R., Ritchie, B., Siegel, J., Simmonds, P., Stanley, S., Tapper, M. L., and von Depka, M. (2006) Clinical perspectives of emerging pathogens in bleeding disorders. *Lancet* **367**, 252-261
8. Kurachi, K., Kurachi, S., Furukawa, M., and Yao, S. N. (1993) Biology of factor IX. *Blood Coagul Fibrinolysis* **4**, 953-973
9. Gil, G. C., Velander, W. H., and Van Cott, K. E. (2008) Analysis of the N-glycans of recombinant human Factor IX purified from transgenic pig milk. *Glycobiology* **18**, 526-539
10. Rehemtulla, A., Roth, D. A., Wasley, L. C., Kuliopulos, A., Walsh, C. T., Furie, B., Furie, B. C., and Kaufman, R. J. (1993) In vitro and in vivo functional

- characterization of bovine vitamin K-dependent gamma-carboxylase expressed in Chinese hamster ovary cells. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 4611-4615
11. Kaufman, R. J., Wasley, L. C., Furie, B. C., Furie, B., and Shoemaker, C. B. (1986) Expression, purification, and characterization of recombinant gamma-carboxylated factor IX synthesized in Chinese hamster ovary cells. *J Biol Chem* **261**, 9622-9628
 12. Bond, M., Jankowski, M., Patel, H., Karnik, S., Strang, A., Xu, B., Rouse, J., Koza, S., Letwin, B., Steckert, J., Amphlett, G., and Scoble, H. (1998) Biochemical characterization of recombinant factor IX. *Semin Hematol* **35**, 11-17
 13. Pipe, S. W., Miao, H., Butler, S. P., Calcaterra, J., and Velander, W. H. (2011) Functional factor VIII made with von Willebrand factor at high levels in transgenic milk. *J Thromb Haemost* **9**, 2235-2242
 14. Orlova, N. A., Kovnir, S. V., Vorobiev, II, and Gabibov, A. G. (2012) Coagulation Factor IX for Hemophilia B Therapy. *Acta naturae* **4**, 62-73
 15. Lissauskas, S. F., Cunha, N. B., Vianna, G. R., Mendes, E. A., Ramos, G. L., Maranhao, A. Q., Brigido, M. M., Almeida, J. O., Baptista, H. A., Motta, F. L., Pesquero, J. B., Aragao, F. J., and Rech, E. L. (2008) Expression of functional recombinant human factor IX in milk of mice. *Biotechnology letters* **30**, 2063-2069
 16. Schnieke, A. E., Kind, A. J., Ritchie, W. A., Mycock, K., Scott, A. R., Ritchie, M., Wilmut, I., Colman, A., and Campbell, K. H. (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278**, 2130-2133
 17. Wang, Y. L., Zhao, S. H., Bai, L., Fan, J. L., and Liu, E. Q. (2013) Expression Systems and Species Used for Transgenic Animal Bioreactors. *BioMed research international*
 18. Dove, A. (2000) Milking the genome for profit. *Nature biotechnology* **18**, 1045-1048
 19. Lai, L., and Prather, R. S. (2003) Creating genetically modified pigs by using nuclear transfer. *Reproductive biology and endocrinology : RB&E* **1**, 82
 20. Paleyanda, R. K., Velander, W. H., Lee, T. K., Scandella, D. H., Gwazdauskas, F. C., Knight, J. W., Hoyer, L. W., Drohan, W. N., and Lubon, H. (1997) Transgenic pigs produce functional human factor VIII in milk. *Nature biotechnology* **15**, 971-975
 21. Velander, W. H., Johnson, J. L., Page, R. L., Russell, C. G., Subramanian, A., Wilkins, T. D., Gwazdauskas, F. C., Pittius, C., and Drohan, W. N. (1992) High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc Natl Acad Sci U S A* **89**, 12003-12007
 22. Lee, H. G., Lee, H. C., Kim, S. W., Lee, P., Chung, H. J., Lee, Y. K., Han, J. H., Hwang, I. S., Yoo, J. I., Kim, Y. K., Kim, H. T., Lee, H. T., Chang, W. K., and Park, J. K. (2009) Production of recombinant human von Willebrand factor in the milk of transgenic pigs. *The Journal of reproduction and development* **55**, 484-490
 23. Lindsay, M., Gil, G. C., Cadiz, A., Velander, W. H., Zhang, C., and Van Cott, K. E. (2004) Purification of recombinant DNA-derived factor IX produced in

- transgenic pig milk and fractionation of active and inactive subpopulations. *J Chromatogr A* **1026**, 149-157
24. Richt, J. A., Kasinathan, P., Hamir, A. N., Castilla, J., Sathiyaseelan, T., Vargas, F., Sathiyaseelan, J., Wu, H., Matsushita, H., Koster, J., Kato, S., Ishida, I., Soto, C., Robl, J. M., and Kuroiwa, Y. (2007) Production of cattle lacking prion protein. *Nature biotechnology* **25**, 132-138
 25. Wojcik, E. G., Van Den Berg, M., Poort, S. R., and Bertina, R. M. (1997) Modification of the N-terminus of human factor IX by defective propeptide cleavage or acetylation results in a destabilized calcium-induced conformation: effects on phospholipid binding and activation by factor XIa. *Biochem J* **323** (Pt 3), 629-636
 26. Hatsuzawa, K., Hosaka, M., Nakagawa, T., Nagase, M., Shoda, A., Murakami, K., and Nakayama, K. (1990) Structure and expression of mouse furin, a yeast Kex2-related protease. Lack of processing of coexpressed prorenin in GH4C1 cells. *The Journal of biological chemistry* **265**, 22075-22078
 27. Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J., and Thomas, G. (1990) Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-beta-NGF in vivo. *The Journal of cell biology* **111**, 2851-2859
 28. Brennan, S. O., and Peach, R. J. (1991) The processing of human proinsulin and chicken proalbumin by rat hepatic vesicles suggests a convertase specific for X-Y-Arg-Arg or Arg-X-Y-Arg sequences. *The Journal of biological chemistry* **266**, 21504-21508
 29. Misumi, Y., Ohkubo, K., Sohda, M., Takami, N., Oda, K., and Ikehara, Y. (1990) Intracellular processing of complement pro-C3 and proalbumin is inhibited by rat alpha 1-protease inhibitor variant (Met352----Arg) in transfected cells. *Biochemical and biophysical research communications* **171**, 236-242
 30. Rehemtulla, A., and Kaufman, R. J. (1992) Preferred sequence requirements for cleavage of pro-von Willebrand factor by propeptide-processing enzymes. *Blood* **79**, 2349-2355
 31. Wasley, L. C., Rehemtulla, A., Bristol, J. A., and Kaufman, R. J. (1993) PACE/furin can process the vitamin K-dependent pro-factor IX precursor within the secretory pathway. *J Biol Chem* **268**, 8458-8465
 32. Drews, R., Paleyanda, R. K., Lee, T. K., Chang, R. R., Rehemtulla, A., Kaufman, R. J., Drohan, W. N., and Lubon, H. (1995) Proteolytic maturation of protein C upon engineering the mouse mammary gland to express furin. *Proc Natl Acad Sci U S A* **92**, 10462-10466
 33. Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E., and Thomas, G. (1994) Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *The EMBO journal* **13**, 18-33
 34. Voorhees, P., Deignan, E., van Donselaar, E., Humphrey, J., Marks, M. S., Peters, P. J., and Bonifacino, J. S. (1995) An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface. *The EMBO journal* **14**, 4961-4975

35. Schafer, W., Stroh, A., Berghofer, S., Seiler, J., Vey, M., Kruse, M. L., Kern, H. F., Klenk, H. D., and Garten, W. (1995) Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. *The EMBO journal* **14**, 2424-2435
36. Jones, B. G., Thomas, L., Molloy, S. S., Thulin, C. D., Fry, M. D., Walsh, K. A., and Thomas, G. (1995) Intracellular trafficking of furin is modulated by the phosphorylation state of a casein kinase II site in its cytoplasmic tail. *The EMBO journal* **14**, 5869-5883
37. Wan, L., Molloy, S. S., Thomas, L., Liu, G., Xiang, Y., Rybak, S. L., and Thomas, G. (1998) PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization. *Cell* **94**, 205-216
38. Molloy, S. S., Thomas, L., Kamibayashi, C., Mumby, M. C., and Thomas, G. (1998) Regulation of endosome sorting by a specific PP2A isoform. *The Journal of cell biology* **142**, 1399-1411
39. Lai, L., and Prather, R. S. (2003) Production of cloned pigs by using somatic cells as donors. *Cloning and stem cells* **5**, 233-241
40. Chauhan, M. S., Nadir, S., Bailey, T. L., Pryor, A. W., Butler, S. P., Notter, D. R., Velander, W. H., and Gwazdauskas, F. C. (1999) Bovine follicular dynamics, oocyte recovery, and development of oocytes microinjected with a green fluorescent protein construct. *Journal of dairy science* **82**, 918-926
41. Zhao, J., Ross, J. W., Hao, Y., Spate, L. D., Walters, E. M., Samuel, M. S., Rieke, A., Murphy, C. N., and Prather, R. S. (2009) Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. *Biol Reprod* **81**, 525-530
42. Van Cott, K. E., Williams, B., Velander, W. H., Gwazdauskas, F., Lee, T., Lubon, H., and Drohan, W. N. (1996) Affinity purification of biologically active and inactive forms of recombinant human protein C produced in porcine mammary gland. *J Mol Recognit* **9**, 407-414
43. Rasband, W. S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2012.
44. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671-675
45. Jagadeeswaran, P., and Sheehan, J. P. (1999) Analysis of blood coagulation in the zebrafish. *Blood Cells, Molecules, and Diseases* **25**, 239-249
46. Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R., and Thomas, G. (1992) Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. *The Journal of biological chemistry* **267**, 16396-16402
47. Anderson, E. D., Molloy, S. S., Jean, F., Fei, H., Shimamura, S., and Thomas, G. (2002) The ordered and compartment-specific autoproteolytic removal of the furin intramolecular chaperone is required for enzyme activation. *The Journal of biological chemistry* **277**, 12879-12890
48. Fischer, K., van der Bom, J. G., Molho, P., Negrier, C., Mauser-Bunschoten, E. P., Roosendaal, G., De Kleijn, P., Grobbee, D. E., and van den Berg, H. M. (2002) Prophylactic versus on-demand treatment strategies for severe haemophilia: a

- comparison of costs and long-term outcome. *Haemophilia : the official journal of the World Federation of Hemophilia* **8**, 745-752
49. UKHCDO. (2003) Guidelines on the selection and use of therapeutic products to treat haemophilia and other hereditary bleeding disorders. *Haemophilia : the official journal of the World Federation of Hemophilia* **9**, 1-23
 50. Dyck, M. K., Lacroix, D., Pothier, F., and Sirard, M. A. (2003) Making recombinant proteins in animals--different systems, different applications. *Trends in biotechnology* **21**, 394-399
 51. Edmunds, T., Van Patten, S. M., Pollock, J., Hanson, E., Bernasconi, R., Higgins, E., Manavalan, P., Ziomek, C., Meade, H., McPherson, J. M., and Cole, E. S. (1998) Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. *Blood* **91**, 4561-4571
 52. Clark, A. J., Ali, S., Archibald, A. L., Bessos, H., Brown, P., Harris, S., McClenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B., and et al. (1989) The molecular manipulation of milk composition. *Genome / National Research Council Canada = Genome / Conseil national de recherches Canada* **31**, 950-955
 53. Zhang, K., Lu, D., Xue, J., Huang, Y., and Huang, S. (1997) Construction of mammary gland-specific expression vectors for human clotting factor IX and its secretory expression in goat milk. *Chinese journal of biotechnology* **13**, 271-276
 54. Yull, F., Harold, G., Wallace, R., Cowper, A., Percy, J., Cottingham, I., and Clark, A. J. (1995) Fixing human factor IX (fIX): correction of a cryptic RNA splice enables the production of biologically active fIX in the mammary gland of transgenic mice. *Proc Natl Acad Sci U S A* **92**, 10899-10903
 55. Van Cott, K. E., Butler, S. P., Russell, C. G., Subramanian, A., Lubon, H., Gwazdauskas, F. C., Knight, J., Drohan, W. N., and Velander, W. H. (1999) Transgenic pigs as bioreactors: a comparison of gamma-carboxylation of glutamic acid in recombinant human protein C and factor IX by the mammary gland. *Genet Anal* **15**, 155-160
 56. McGrath B.M., W. G. (2005) *Directory of therapeutic enzymes.*, Taylor & Francis, N.Y.

CHAPTER 4

SCALED-UP PURIFICATION OF RECOMBINANT HUMAN COAGULATION FACTOR IX FROM MILK PRODUCED IN MAMMARY GLAND OF TRANSGENIC PIG

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ABSTRACT

Hemophilia B is a congenital bleeding disorder caused by the deficiency of functional coagulation factor IX (FIX). The current treatment is restricted to the protein replacement, including the on-demand or prophylaxis. In 2012, approximately 1 billion FIX concentrates were consumed by 28,008 hemophilia B patients worldwide. However, still about 80% patients, mostly in developing countries, receive inadequate or no treatment because of unavailable or unaffordable FIX concentrates. Consider the plasma derived FIX transmitting blood-borne pathogen, recombinant human FIX is recommended. More than 99% of consumed FIX concentrates was recombinant in United States. Our lab reported expressing recombinant human FIX (tg-FIX), 2-3 g/L with 10-20% active FIX, in milk by mammary gland of transgenic pig with lab scale purification developed. In this study, large scale purification protocol was established. The brand new camelid IgG based immunoaffinity chromatography efficiently in capture the whole tg-FIX population in the milk and the high pressure liquid chromatography effectively separate the active tg-FIX from non-active tg-FIX and activated tg-FIX subpopulation. 4 chromatography isolated active tg-FIX with similar specific activity as plasma derived FIX.

INTRODUCTION

Hemophilia type B (Christmas disease) is a congenital bleeding disorder caused by the deficiency of coagulation factor IX (FIX). 28,008 patients has been diagnosed with hemophilia B in 2012 worldwide(1). The ideal treatments for hemophilia B patients are prophylactically, which significantly reduces complications and improves the quality of life (2), or the on-demand replacement of blood FIX when bleeding episodes occur (3). In 2012, about 1 billion IU of FIX concentrates was consumed worldwide(1). However,

actual need of the FIX supply could be much more than the reported number, since 70-80% patients received inadequate or no treatment because of the high price of the FIX concentrates (4). Up-to-date, 14 plasma derived (pd-) and 1 recombinant human FIX concentrates are licensed and commercially available on the market (3). Consider the transmission of blood-borne diseases and infections, the recombinant human therapeutic protein was recommended(5). So, producing the safe recombinant human FIX in an economically is required.

The FIX gene is located on the X-chromosome at q27 and expressed in hepatocytes in a highly tissue-specific manner (6,7). The nascent FIX is expressed in a pre-pro form and conducted multiple post-translational modifications (PTMs). These PTMs include the removal signal peptide, vitamin-K dependent gamma-carboxylation of first 12 glutamic acid(8,9), pro-peptide removal(10,11), glycosylation(12-15), beta-hydroxylation of Asp-64(16), sulfation of Tyr-155(17) and phosphorylation of Ser-158(18-20). The complexity of theses PTMs could be performed by certain type of mammalian cells. Chinese hamster ovary (CHO) cell, for example, was used for expression the only one recombinant human FIX (BeneFIX) on the market approved by FDA. It was characterized having limitations on performing those PTMs. Unlike the plasma-derived human FIX, BeneFIX contains a mixture of subpopulations with 10, 11 and 12 γ -carboxyglutamic acids, no phosphorylation at Ser158, and only 15% sulfation of Tyr155(21).

Currently, mammary gland of transgenic livestock is a promising bioreactor for producing recombinant human proteins economically. Considering the high cell density (about 10^9 cell mL⁻¹) of the gland tissue, at the production of recombinant proteins could be 2-3 orders of magnitude greater than density of mammalian cells in steel bioreactors

(22). Additionally, the milk production is high, for example, a pig produces about 200-400 L per year (23,24). However, not all of them are suitable for producing active FIX efficiently. No active FIX had been detected in the transgenic sheep milk(25). Zhang et al reported that > 90% recombinant protein in milk appeared to be a gamma-glycosylated and biologically active but the expression level was only 13.7 ng/mL (26). In contrast, the transgenic pig, was reported expressing 2-3 g/L recombinant human FIX (tg-FIX) in the milk and 10-20% population with bio-activity(27). Pig is not sensitive to prions; while the bovine PRNP gene required for the development of prion diseases(28). In addition, cost of establishing a transgenic commercial production herd could be approximately a sixth of building a commercial cell-culture facility (29). So mammary gland of transgenic pig could produce the safe recombinant FIX, economically, to meet the need for the hemophilia B patient worldwide. In addition, with the drop of the cost of FIX concentrates, oral administration of FIX concentration will be an option to replace the traditional discomfort, potential for opportunistic infection intravenous.

After active tg-FIX successfully expressed in the pig milk, the downstream processing is important for achieving pure active FIX. However, milk is a challenging feedstock for purification because of the large amount native proteins, including the casein (~ 30g/L), whey proteins (lactoferrin, α/β -lactalbumin), and serum-passover proteins (serum albumin, immunoglobulins, transferrin) and its natures as a multiple-phase mixture, including fat, solid and aqueous phases [paper in draft]. Our lab previously made progress in developing purification steps by using the heparin-affinity chromatography combined with ion-exchange chromatography to isolate the active tg-FIX subpopulation from the transgenic pig milk (27). In this study, the more efficient camelid IgG (30)

based immunoaffinity chromatography was applied to capture the whole tg-FIX population, and the high pressure liquid chromatography size exclusion chromatography (HPLC-SEC) was applied to separate active tg-FIX from the low active and activated tg-FIX subpopulation. In addition, the scaled up protocol was established to meet the large quantity of tg-FIX.

MATERIALS AND METHODS

All buffer components were purchased from VWR International LLC (Radnor, PA), Thermo Fisher scientific (Waltham, MA), or Sigma (St. Louis, MO) unless otherwise stated. Reference pd-FIX (Mononine, CSL Behring, USA) was a kind gifts from James Brown (Lincoln, NE). These stocks of were expired for clinical use but when used in experiments exhibited full procoagulant activity by one stage clotting assay. It was reconstituted in 20 mM calcium TBS (20 mM Tris, 0.15 M NaCl) pH 7.4 or 10 mM EDTA TBS pH 7.4 and then characterized by one stage clotting assay and SDS-PAGE to assure homogeneity and biological activity before using in the plasmin degradation studies made here.

Transgenic Milk Collection and Storage

Transgenic pigs were generated as described previous [paper in draft]. In this study, transgenic milk with recombinant human FIX expressed (tg-FIX milk) and transgenic milk with recombinant human furin co-expressed with recombinant human FIX (tg-furin-FIX milk) were both investigated. The transgenic swine milk was collected as described with modification whole milk samples were directly stored at -80 °C without treatment of EDTA or any other protease inhibitors before storage.

Cleavage of Pro-peptide of Pro-FIX by Tg-furin *in vitro*

Whole tg-furin-FIX milk sample was first added to heparin Sepharose FF (GE Healthcare Bio-Sciences AB, Sweden) resin at 1:1 (v/v) ratio and incubated for 30 min. The supernatant was collected (FIX depleted) and was then incubated with the whole tg-FIX milk at 1:2 or 1:4 (v/v) ratio for 1.5 hour at 4 °C. For the scaled up preparation, the whole tg-furin-FIX milk was directly incubated with whole tg-FIX milk at 1:4 (v/v) ratio for 2 hour at room temperature. All above milk samples were not treated with EDTA and any protease inhibitors.

Clarification of Transgenic Milk

Milk samples, pro-peptide was processed, were mixed with 0.43 M Citrate, pH 6.2 at 1:1 (v/v) ratio (No PIs treated) or 0.43 M Citrate, 200 mM EDTA, 80 mM ϵ -aminocaproic acid (EACA), 40 mM Benzamidine, pH 6.2 at 1:1 (v/v) ratio (with PIs treated), then centrifuged for 2 hours at 9000 rpm at 4 °C (Sorvall Evolution RC centrifuge with SLC-4000 rotor, Thermo). The top fat layer and the bottom casein pellet were removed and the middle supernatant was further filtered with cheesecloth.

Capture of Whole FIX Population in Transgenic Milk by FIX Select

The milk samples were loaded on the FIX select, camelid Monoclonal antibody anti-human FIX coupled to Sepharose fast flow (GE), XK26/20 column (GE healthcare, Sweden), The flow rate is 6mL/min. And whole FIX population was eluted by elution buffer (2M MgCl₂). The chromatography was performed at room temperature.

Isolation of High Acidic FIX from FIX Select Eluate

This method was adapted from previous (27). The total FIX population (FIX select elution product) was exchanged into the equilibrium buffer (20mM imidazole, 0.1M Ammonium acetate, pH 7.0). The FIX sample was loaded onto Q Sepharose fast flow, XK26/20 column (GE healthcare, Sweden). The flow rate is 4 ml/min. The low acidic FIX population was washed out by increased [Ammonium acetate] to 537 mM, and the high acidic FIX was eluted at [Ammonium acetate] at 800 mM. The Q eluate (EL) was further exchanged into the ceramic hydroxyapatite (CHT) equilibrium buffer (20mM potassium phosphate, pH 7.2) and loaded on the CHT Type I 40um (BioRad, Richmond, CA) XK16/25 column (GE healthcare, Sweden). The flow rate is 3mL/min. The low acidic FIX was removed in the wash step by at 65 mM potassium phosphate. Then high acidic FIX population was eluted at 200 mM potassium phosphate. Latter portion (about 80%) of EL (CHT EL 2) was collected. All buffer exchanges of tg-FIX samples were performed by using the tangential flow filter (TFF) 10 kDa MWCO (Pall Corporation, Port Washington, NY).

Separate Active FIX from High Acidic FIX population by High

Performance/Pressure Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC)

The high acidic tg-FIX was exchanged into SEC running buffer (20 mM Tris, 200 mM NaCl, 10 mM CaCl₂, pH 7.4) by using the Minimate tangential flow filtration systems with 10 kDa molecular weight cut off (MWCO) membrane (Pall Corporation, Port Washington, NY) or Amicon tube (10 kDa MWCO) (EMD Millipore, Billerica, MA). For analytical run (≤ 1 mg FIX), FIX sample was loaded on a TSK gel G3000SW_{XL}, 7.8

mm ID x 30 cm, 5 μ m (particle size), equipped with a guard column, 6 mm ID x 4 cm, 7 μ m (particle size), (Tosoh Bioscience, LLC). The flow rate was 0.5 ml per min and duration of the run was 45 min with all major peaks eluting before 35 min. For large preparations (up to 10 mg FIX), FIX sample was loaded on a TSK gel G3000SW with 21.5 mm ID x 60 cm, 13 μ m (particle size), equipped with a guard column, 8 mm ID x 4 cm, 10 μ m (particle size), (Tosoh Bioscience, LLC). The flow rate was set at 3 ml/min and duration of the run was 90 min with all major peaks eluting before 60 min. The chromatography were performed by using Knauer Smartline chromatography apparatus, including Manger 5000, Pump 1000 and UV Detector 2600 (Berlin, Germany), equipped with a Gilson FC 204 fraction collector (Middleton, WI). Both columns were equilibrated and run with 20 mM Tris base, 200 mM NaCl, 10 mM CaCl₂, pH 7.0. The fractions of each peak were pooled together for further analysis.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were analyzed by SDS-PAGE stained with colloidal Blue gel stain (Invitrogen, Carlsbad, CA, USA) using Invitrogen 12% Bis-Tris Novex acrylamide precast gels and the Invitrogen Surelock XL apparatus (200 Volts for 1 hour). Briefly, samples were mixed with 4x LDS sample buffer (Invitrogen) and deionized water followed by heating at 74 °C for 10 min. For reduced gels, samples were mixed with 10x reducing agent (Invitrogen) prior to heating.

Western Blot

The gels were electro-blotted onto poly vinylidene fluoride (PVDF) membranes using a BioRad Transblot SemiDry Transfer Cell (Richmond, CA) for 30 min at 25 volts. For total FIX, including all FIX species, blots were probed by rabbit polyclonal antibody (Pab)

anti-F9 (F0652, Sigma-Aldrich St. Louis, MO); The furin was detected by using rabbit Pab anti-furin (476-490) (F8806, Sigma-Aldrich St. Louis, MO); The mouse monoclonal antibody (Mab), PROFIX 3D2.6H12 (Green Mountain Antibody, Burlington, VT), was used in probing pro-peptide of FIX. The secondary antibody (HRP-conjugated) anti-rabbit (A9169) and mouse (A4416) IgG (Sigma-Aldrich St. Louis, MO) was used to detect the primary antibody respectively. The chromogenic DAB substrate (Thermo, Rockford, IL) was used in color developing.

Quantification of Recombinant Human Factor IX (rF9) and Recombinant Human Pro-peptide attached Factor IX (pro-rF9) in Transgenic Milk

Western blot was performed as described before. Mononine, as calibrator, was loaded along with the transgenic milk samples (triplicated). The concentration mononine was determined by measuring the optical density at 280 nm in a 1 cm quartz cuvette and using an extinction coefficient, $\epsilon^{1\%} = 13.4$. The amount of tg-FIX was then calculated by using the standard curve created by the calibrators. The pictures of the blots were analyzed by using the Image J(31,32).

Quantification of FIX by ELISA

The procedure was modified from previous literature (33). Enzyme immunosorbent assay plates (Greiner Bio-One, Monroe, NC) were coated overnight at 4°C with 100 μ L at 2 μ g/ml mouse anti-human factor IX IgG (Haematologic Technologies, Inc., Essex Junction, VT) per well in carbonate buffer. Factor IX samples at 100 μ L/well including those for the standard curve (0.98-200 ng/mL) were incubated for 2 hours at 25°C. Then sheep anti-human factor IX IgG conjugated with horseradish peroxidase (HRP; Affinity Biologicals, Hamilton, Canada) (100 μ L/well at 1 μ g/ml in blocking buffer) was added

and incubated for 1 hour at 25 °C. After washing, 150 µL/well 1-step ABTS (Thermo, Rockford, IL) was added and color was allowed to develop up to 45 minutes and measuring absorbance at 405 nm.

Quantitation of Total Factor IX Activity

Total FIX activity was determined using the activated partial thromboplastin time (aPTT) assay (34). Briefly, 50µl each of PTT Automate 5 reagent (Diagnostica Stago, Inc., Parsippany, NJ, USA), factor IX deficient plasma (George King Bio-Medical, Overland Park, Kansas, USA), and a sample of interest were added to a cuvette and incubated at 37 °C for 3 min. 50 µL of 25mM CaCl₂ (Sigma) was then added and the clotting time was measured using the STart Hemostasis Analyzer (Diagnostica Stago, Inc., Parsippany, NJ). The normal human plasma (Diagnostica Stago, Inc., Parsippany, NJ) was used as the standard, assigning 1 unit of FIX clotting activity per milliliter of plasma. The concentration of purified tg-FIX was determined by measuring the optical density at 280 nm in a 1 cm quartz cuvette and using an extinction coefficient, $\epsilon^{1\%} = 13.4$.

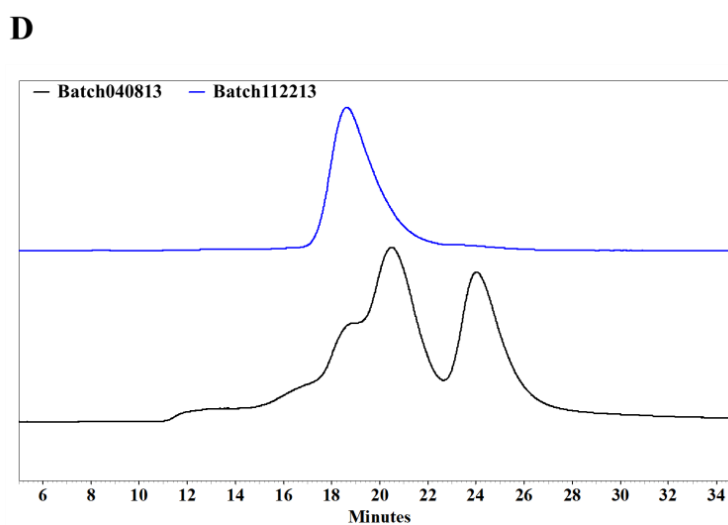
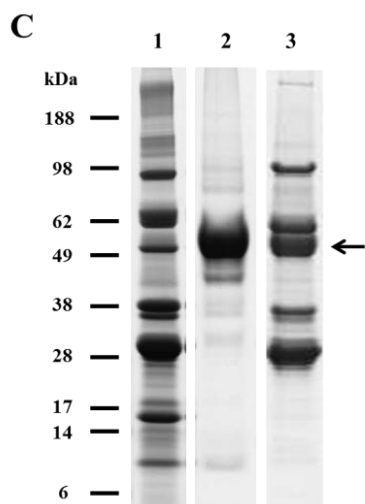
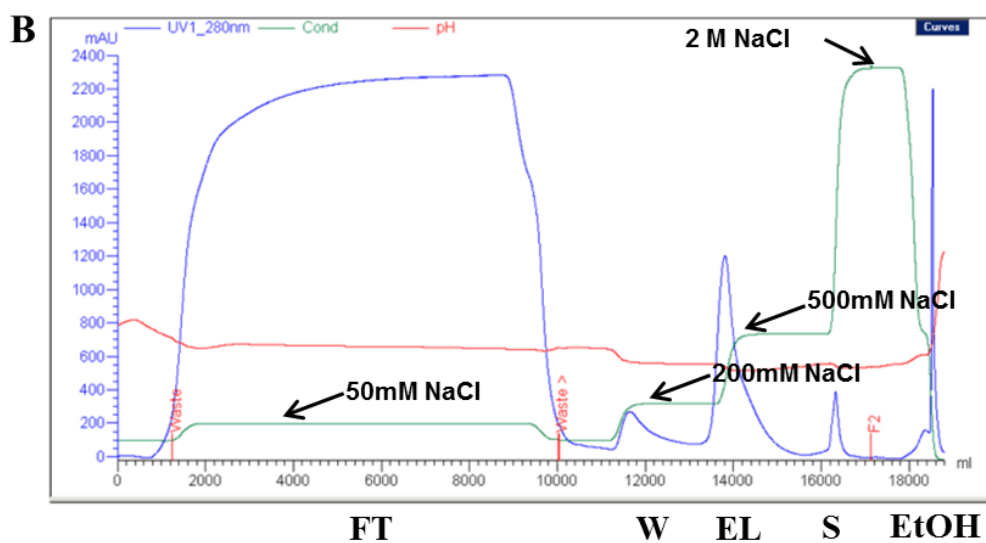
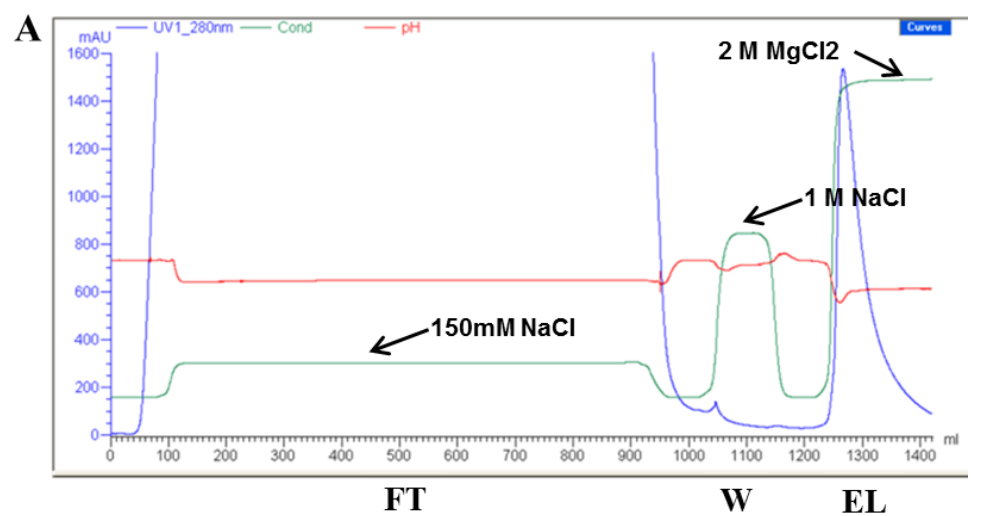
NH₂-terminal Sequencing of the Plasmin Cleavage Products of FIX

Amino acid sequencing was performed at the University of Nebraska Medical Center, Protein Structure Core Facility. Protein samples were subjected to SDS-PAGE and transferred to PVDF as described above. The membrane was stained with Colloidal Blue and bands excised and subjected to Edman degradation using Applied Biosystems Procise protein sequencer, a Hitachi 8800 Amino Acid Analyzer and a Michrom MAGIC HPLC equipped with a Diode Array Detector.

RESULTS

Capture Total TG-FIX Population in Transgenic Milk by FIX Select

The camelid IgG anti-FIX based immunoaffinity column was used in capture whole tg-FIX population in the transgenic milk. A sharp peak in the elution step followed the huge amount unbound flow through (FL) and tiny amount of wash (Fig.1A); the recovery of tg-FIX by using FIX select column was ~ 85% (Table 3). The heparin affinity chromatography, widely used in purifying FIX from both plasma, mammalian cell culture and transgenic milk, required additional wash step to removed caseins and other impurities in milk; the quantity of these heparin binding impurities was larger than the tg-FIX population and taking large amount of binding capacity of the column (Fig. 1B). By comparing the BCA results (total protein in sample) and FIX specific ELISA (tg-FIX in sample), FIX select eluate (EL) (batch112213) was about 95% tg-FIX (data not shown). The non-reduced SDS-PAGE showed FIX select EL was much purer (Fig. 1C, lane 2) than EL from heparin affinity chromatography (Fig. 1C). Large quantity of porcine plasmin was detected in the milk sample. No plasmin signal could be detected in the EL sample after the chromatography (Fig. 1E), which indicate the efficiency of FIX select in removing protease in the milk sample. The FIX select EL was then analyzed by the HPLC-SEC, it had only peak 1 compared to 3 peaks (larger peak 2 and 3) of high acidic tg-FIX (batch040813) (Fig. 1D). The FIX specific activity of the FIX select EL was 174 ± 10 U/mg (Avg. \pm SD, n=3), looks “normal” compared to the 200 U/mg of pd-FIX. However, this activity was contributed mostly by the activated tg-FIX population (SEC pool 3, 2288 ± 204 U/mg) (Avg. \pm SD, n=3) (Table 1)



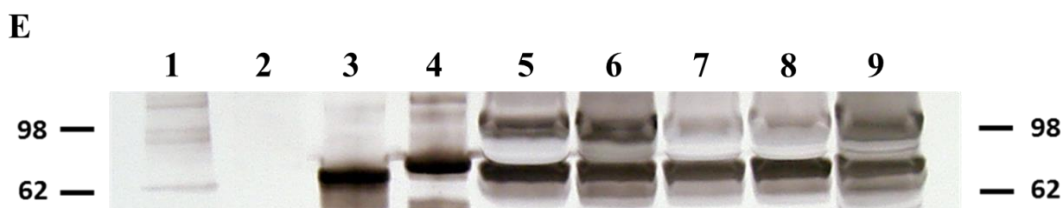


Fig. 1. Comparison of FIX Purification by FIX Select and Heparin. **A.** Chromatograph of FIX select run of transgenic milk sample (K108). **B.** Chromatograph of Heparin Hyper D run of transgenic milk sample (k82, k89, k92 and k102 pool). FL: Flow through of the sample; W: Wash; EL: Elution; Strip: 2 M NaCl; EtOH: Ethanol. The blue line indicate the protein (OD_{280}), the green line indicate the conductivity (mS) and the red line indicate the pH. **C.** Non-reduced SDS-PAGE of total FIX population captured by FIX select or Heperin Hyper D. Lane 1: Clarified milk; Lane 2: Eluate from FIX select run; Lane 3: Eluate from Heparin Hyper D run. The arrow indicates the FIX in eluate of Heparin Hyper D. **D.** Comparison of SEC profiles of FIX select EL (batch112213) and CHT EL 2 (batch040813). **E.** Non-reduced Western blot detecting porcine plasmin in purified tg-FIX sample and transgenic milk sample. Lane 1, tg-FIX111413 (FIX select EL), 20 μ g; lane 2, tg-FIX040813 (CHT EL2), 10 μ g; lane 3, human plasmin (83 kDa), 1 μ g; lane 4, human plasminogen (88 kDa), 1 μ g; lane 5-9, transgenic milk K75, K101, R175&179, R180 and R1014 lactation pool, diluted 1:1 (v/v) and skimmed, 10 μ L (about 10 μ g tg-FIX).

Isolation of High Acidic Tg-FIX from FIX Select Eluate

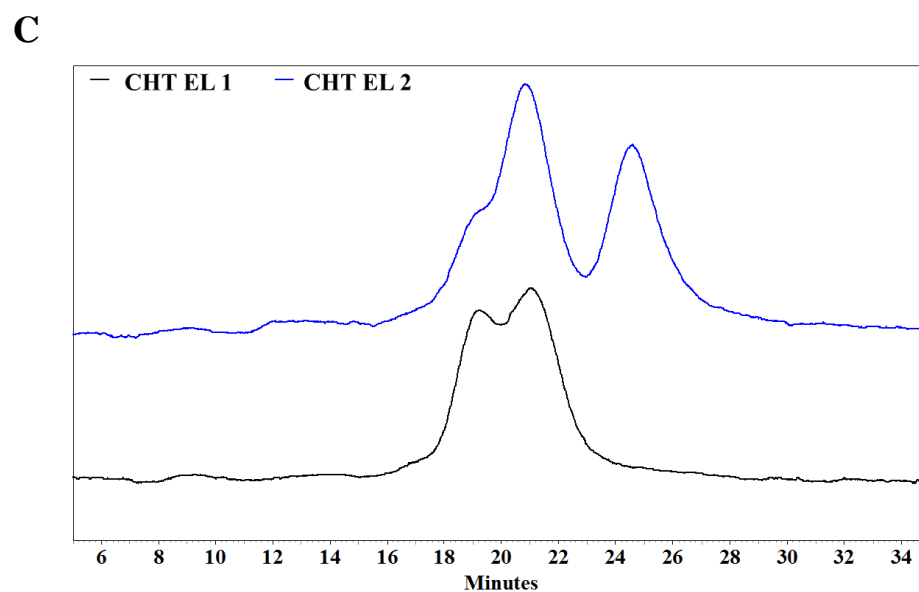
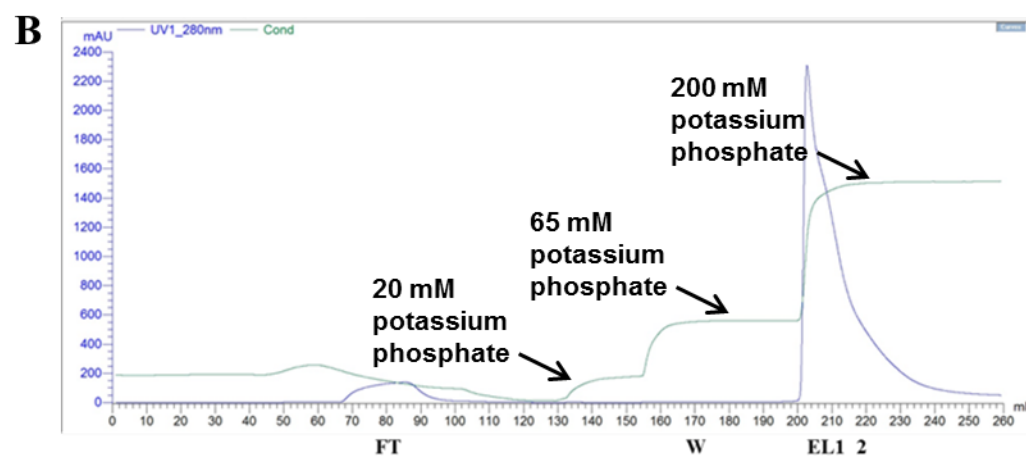
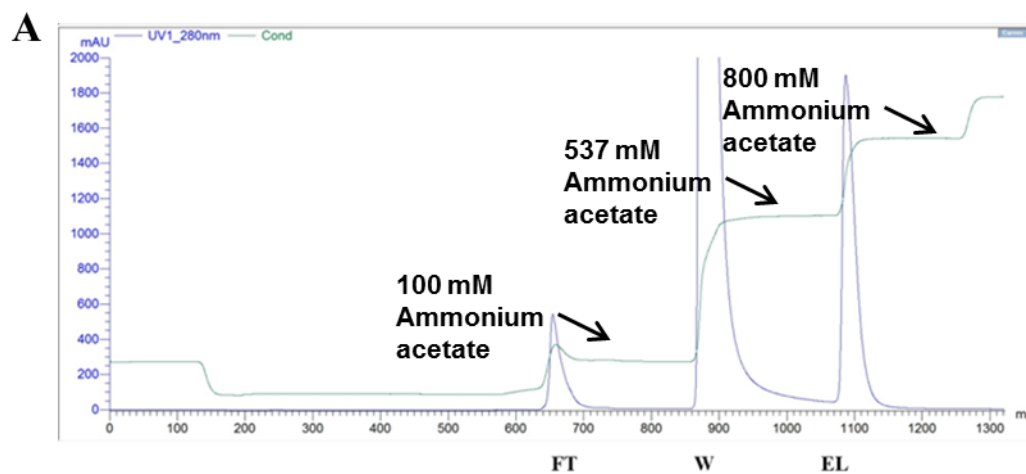
Q sepharose fast flow column was used in isolating high acidic, active, tg-FIX population and the methods were adapted from gradient (27) to step wash (Fig. 2A). The Q EL (batch 091713) had 4 times more FIX specific activity than the FIX select EL (batch112213), and an additional lower band (45 kDa) than the wash fraction (Fig. 2D, lane 3-4). We noticed the increasing in activity was from both the fraction of active tg-FIX, SEC pool 2, and activated tg-FIX (tg-FIXa), SEC pool 3 (Table 1).

The Ceramic Hydroxyapatite (CHT) chromatography was performed after Q to further enrich the high acidic tg-FIX. The high acidic tg-FIX subpopulation was enriched in latter 80% EL (EL 2), whose peak 1 on the HPLC-SEC was less than CHT EL 1 (Fig. 2B, 2C). Like the Q EL, CHT EL 2 had more 45 kDa band on the non-reduced gel (as shown

in the Q EL) (Fig. 2D, lane 5 and 6). SEC pools of CHT ELs loaded on reduced SDS-PAGE was analyzed by N-terminal amino acids sequencing, majority of the pool 3, which only shown in EL 2, was tg-FIXa (the 28 kDa band showed a.a. sequence of VVGGE, indicating the heavy chain of tg-FIXa), while no tg-FIXa was detected in CHT EL 1 (Fig. 2E), This was also confirmed by the activity assay (Table 1 and 2).

Batch	Samples	Mass Percentage %	Specific Activity (U/mg)
112213	FIX Select Eluate	100	174 ± 10
	SEC Pool 1	55	1 ± 0
	SEC Pool 2	43	9 ± 1
	SEC Pool 3	1	2288 ± 204
91713	Q Eluate	100	714 ± 7
	SEC Pool 1	21	9 ± 1
	SEC Pool 2	43	107 ± 7
	SEC Pool 3	36	2755 ± 182
40813	CHT Eluate	100	2351 ± 335
	SEC Pool 1	16	No Activity
	SEC Pool 2	41	165 ± 9
	SEC Pool 3	43	4284 ± 728
42313	CHT Eluate	100	1471 ± 147
	SEC Pool 1	18	No Activity
	SEC Pool 2	48	138 ± 8
	SEC Pool 3	34	3753 ± 278

Table 1. Comparison of FIX Purification Samples by using Analytical HPLC-SEC. About 700 µg FIX eluate sample of each batch (purified by using different procedures) was loaded on HPLC-SEC and the fractions were pooled (SEC pool 1, 18-19.5 min; SEC pool 2, 20-22 min; and SEC pool 3, 22.5-27 min) and calculated FIX specific activity. All above batches were purified from the transgenic milk (K108). The mass percentage was calculated based on amount of FIX in each fraction. The specific activity of control plasma derived FIX was 211 ± 9 U/mg (Avg. ± SD, n=3).



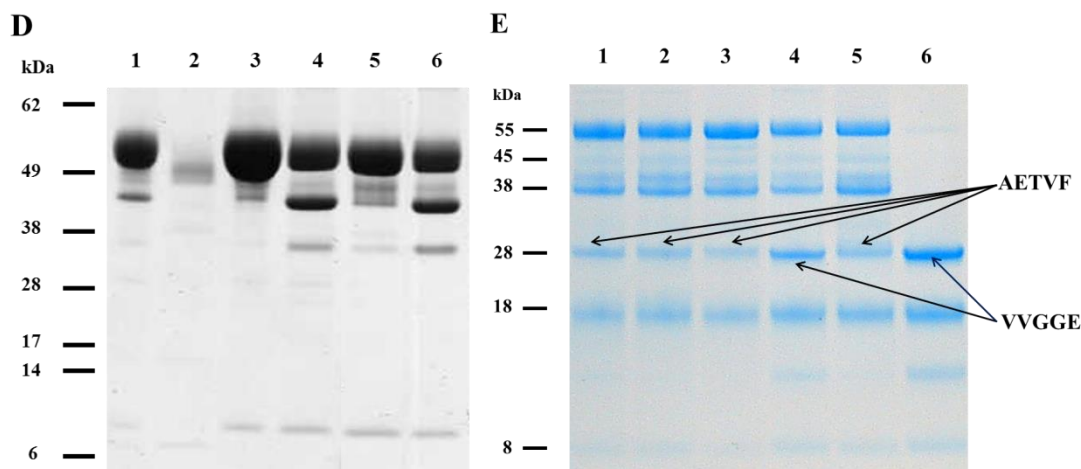


Fig. 2. Isolation of High Acidic FIX by Using Ion-exchange Chromatography. **A.** Q sepharose FF run. **B.** CHT run. Flow through (FT), wash (W) and eluate (EL) were labeled. The blue line indicate the protein (OD_{280}), the green line indicate the conductivity (mS). **C.** Comparison of SEC profile of CHT EL 1 and 2 (batch031313). **D.** Non-reduced SDS-PAGE of FIX purified from Q and CHT (batch040813). Lane 1: FIX select EL; Lane 2: Q FT; Lane 3: Q wash; Lane 4: Q EL; Lane 5: CHT EL 1; Lane 6: CHT EL 2. **E.** Reduced SDS-PAGE of FIX samples (Batch031313). Lane 1: CHT EL 1; Lane 2: CHT EL 1 SEC pool 1; Lane 3: CHT EL 1 SEC pool 2; Lane 4: CHT EL 2; Lane 5: CHT EL 2 SEC pool 2; Lane 6: CHT EL 2 SEC pool 3. The 28 kDa band of each lane was sequenced and the N-terminal first 5 amino acid sequence listed.

Separate Active Tg-FIX from High Acidic Tg-FIX population by High

Performance/Pressure Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC)

Although the FIX had been purified into a highly acidic tg-FIX subpopulation, the FIX material only contained ~40% active tg-FIX analyzed by HPLC-SEC. The CHT EL 2 (batch040813) had about 10% non-active tg-FIX species and the other ~40% was mixture of tg-FIXa and degraded tg-FIX (mostly tg-FIX γ) (Table 1). To scale up the separation of active tg-FIX from no-active and tg-FIXa/degraded tg-FIX, TSK gel G3000SW with 21.5 mm ID x 60 cm L, 13 μ m (particle size) at higher throughputs was applied in purification procedure. We noticed resolution in separation of peak 2 and 3 was better in using the

processing HPLC-SEC (Fig.3). However, with 10-folds higher load on the HPLC-SEC, approximately 30% of total loaded tg-FIX came out before the regular 3 peaks, which was not present in analytical runs and was considered as the aggregation of tg-FIX sample.

CHT Eluate (Mass Percentage)	SEC Pool		
	Specific Activity (U/mg) (Mass Percentage)		
	1	2	3
1 (8%)	11 \pm 1 (31.6%)	122 \pm 7 (68.4 %)	N/A
2 (73%)	25 \pm 3 (18 %)	155 \pm 13 (46 %)	3203 \pm 890 (37 %)

Table 2. Comparison of CHT eluate 1 and 2 SEC pools (Batch031313) (Avg. \pm SD, n=3).

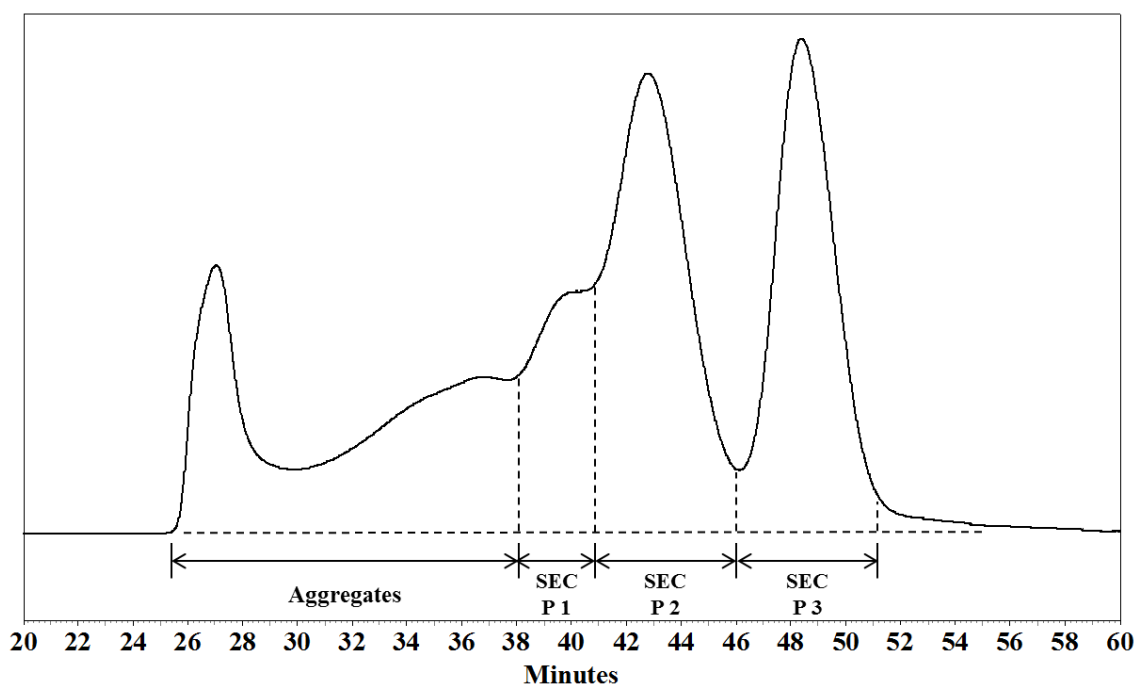


Fig. 3. Processing HPLC-SEC Profile of CHT EL 2 (batch031313). 10 mg FIX CHT EL 2 (~2mg/ml) was loaded on the TSK gel G3000SW (21.5 mm ID x 60 cm L) with running buffer (20 mM Tris, 200 mM NaCl, 10 mM CaCl₂, pH 7.0). The Flow rate was 3 ml/ min. The run was 90 min. All peaks came out before 60 min. Fractions were pooled as indicated on the figure.

Inefficiency in Separating Tg-proFIX from Active Tg-FIX

We previously reported the inefficiency in processing of pro-peptide of tg-proFIX (pro-peptide attached tg-FIX) by endogenous porcine furin in mammary gland [paper in draft].

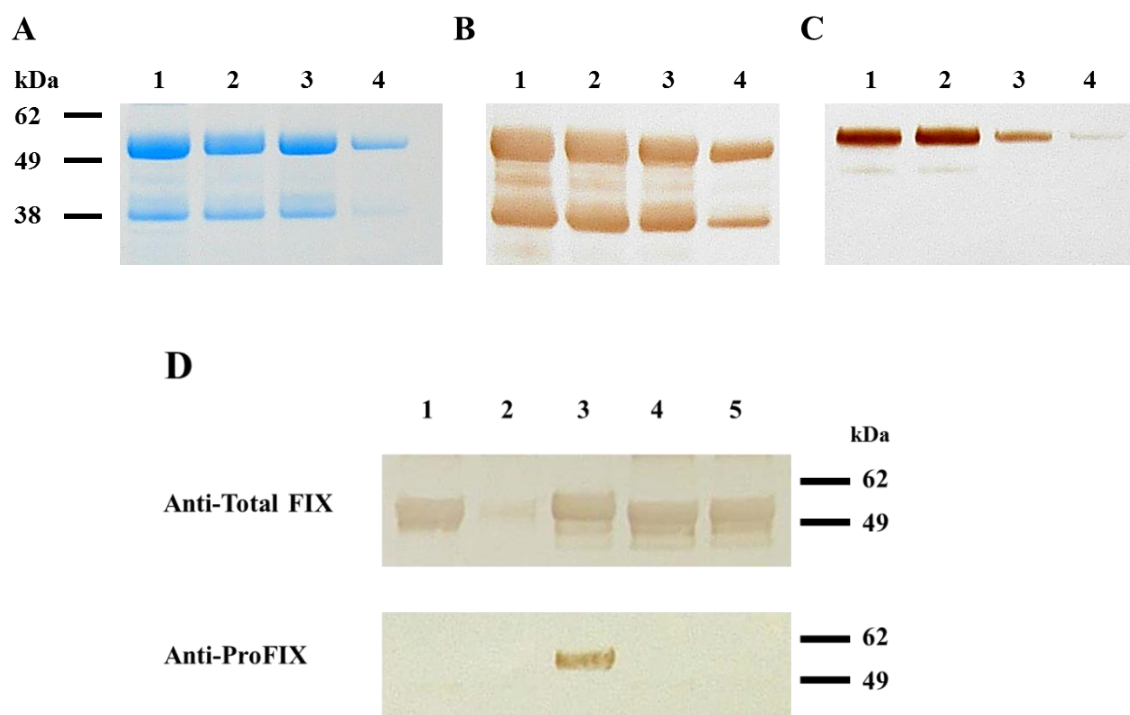


Fig. 4. Reduced SDS-PAGE and Western blots of FIX purified by HPLC-SEC. **A.** Reduced SDS-PAGE; **B.** Reduced Western blot developed by using antibody anti-total FIX; **C.** Reduced Western blot developed by using antibody anti-proFIX. Lane 1: High acidic FIX (4 μ g, Q Sepharose eluate, batch091713); Lane 2: SEC pool 1 of high acidic FIX (2 μ g, non-active FIX); Lane 3: SEC pool 2 of high acidic FIX (2 μ g, active FIX); Lane 4: SEC pool 3 of high acidic FIX (1 μ g, activated FIX or FIX γ) (SEC pool 1, 18-19.5 min; SEC pool 2, 20-22 min; and SEC pool 3, 22.5-27 min). The concentration of each FIX sample was measured by optical density with wavelength = 280 nm, $\epsilon^{1\%} = 13.4$. **D.** Western blot of removal of propeptide of FIX by furin co-expressed in the transgenic milk. Lane 1: Furin co-expressed FIX milk (0.8 μ L, R175); Lane 2: FIX depleted furin co-expressed FIX milk (0.8 μ L); Lane 3: FIX milk (0.8 μ L, K96); Lane 4: Sample of furin co-expressed FIX milk incubated with FIX milk, 1:2 (v/v) at 4°C for 1.5 hour; Lane 5: Sample of furin co-expressed FIX milk incubated with FIX milk, 1:4 (v/v) at 4°C for 1.5 hour. All samples were whole milk without EDTA or protease inhibitor treatment.

We noticed the ion-exchange chromatography could not separate the non-active tg-proFIX from the active tg-FIX. The high acidic tg-FIX (batch 091713, without pro-peptide cleavage) contained a large portion of tg-proFIX (Fig. 4, lane 1). HPLC-SEC failed in separating tg-proFIX, 2 kDa difference, from FIX zymogen. All three SEC pools contained tg-proFIX (Fig. 4, lane 2-4), which decreased the activity of final FIX samples (batch 091713) (Table 1). To eliminate tg-proFIX and recover the activity by converting inactive tg-proFIX to active zymogen, the pro-peptide needs to be processed before all purification steps. Tg-proFIX was converted into tg-FIX after incubated with tg-furin in a lab scale experiment (Fig. 4D, lane 4 and 5). For large preparation, the FIX milk (K108) was treated with the tg-furin-FIX milk (R185) at 1:4 ratio at room temperature for 2 hours before the FIX select step. No tg-proFIX was detected in the final produce (data not shown), and the FIX specific activity of tg-FIX sample (batch040813 SEC pool 2) was increased (Table 1).

Minimize the tg-FIX Degradation during the Purification

We previously reported that the major protease in transgenic swine milk, plasmin, degrades the tg-FIX [paper in draft]. In addition, some other protease, such as trypsin, might be also involved in the proteolysis of tg-FIX in transgenic milk (35). The ϵ -aminocaproic acid (EACA), which is the specific inhibitor of plasmin, and benzamidine, inhibiting trypsin, was used to prevent the degradation of tg-FIX in milk. SEC profile of batch 040813, which was not treated PIs, has larger peak 3 (43%), than the batch 042313 (34%), which was treated PIs (Fig. 5, Table 1), although both batches were used the same transgenic milk sample. The activity assay showed batch 040813 has more tg-FIXa

(Table 1). So, introducing PIs helped decreasing the activation/degradation of tg-FIX in the milk sample.

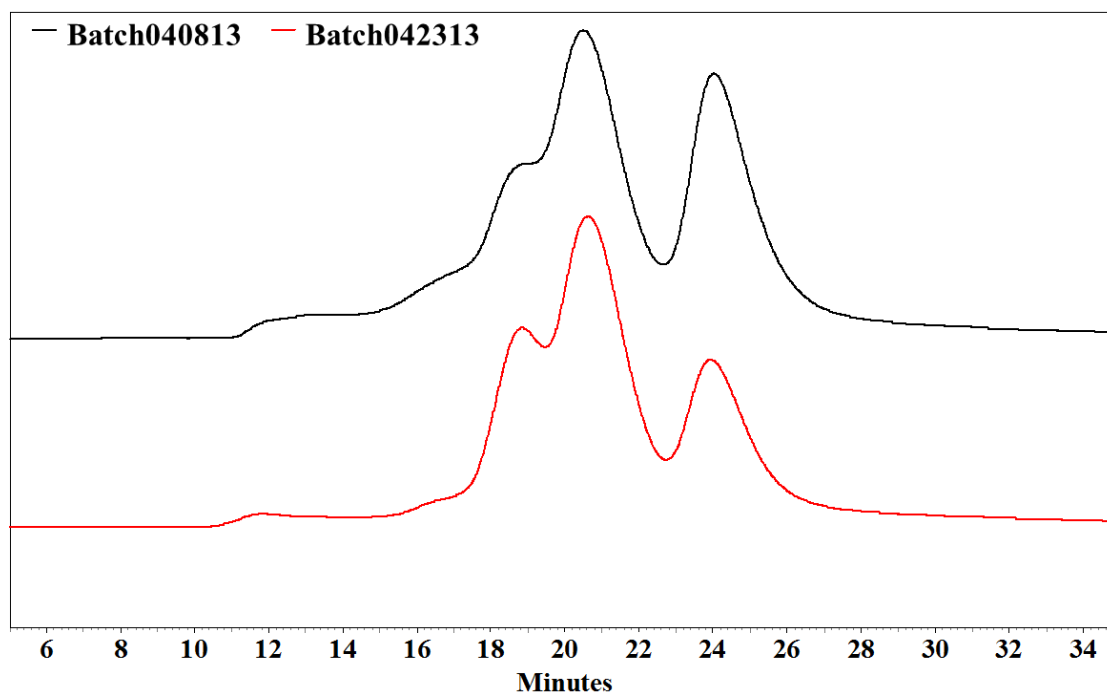


Fig. 5. Comparison the SEC profile of Purified FIX Sample with or without treated by protease inhibitors. The upper one was not treated by PIs (batch 040813). The lower one was treated by PIs (batch 042313). Both batches were from same milk and using the same purification procedures.

CONCLUSION

The mammary gland of transgenic swine has the advantages, including the high cell density in the gland tissue, the high volume of milk produced per swine, the high recombinant protein yield and its capability to perform certain PTMs for recombinant human coagulation proteins, making it a promising bioreactor for producing FIX economically. Biologically active recombinant human coagulation factors have been successfully produced in pig milk and reported by several studies, such as human F8 (36),

protein C (37) , VWF(38) and F9 (27,39). Additionally, the capital investment is substantially less than the traditional steel bioreactor for both mammalian cells and bacteria. However, the complexity of the milk, such as the large quantity of the native proteins and multiple phase nature, make it more of a challenge for downstream processing than current mammalian and bacteria system.

Purification Step	Batch031313	Batch040813
	FIX in Eluate (mg) (Overall Yield %)	FIX in Eluate (mg) (Overall Yield %)
Clarified Milk	1400 (100)	1400 (100)
FIX Select	1171 (84)	1180 (84)
Q Sepharose	136 (10)	169 (12)
CHT	88 (6)	92 (7)
SEC	32 (2)	38 (3)

Table 3. Purification Table of Active FIX from Transgenic milk. Both batches were purified from the same pig milk, batch 031313 was using the lactation day 16-19 pool, and batch040813 was used the lactation day 5-14 pool.

The new camelid IgG anti-FIX immuno-affinity chromatography, more efficient than the traditional heparin affinity column, captured whole FIX population with about 84% yield and close to 100% purity (Table 3). The efficiency in purifying tg-FIX from impurities is also important in decreasing the proteolysis of tg-FIX by proteases, e.g. porcine plasmin, in the milk. In addition, no acidic pH was needed in elution steps that are required by traditional immunoaffinity chromatography. The ion-exchange chromatography, Q sepharose and Ceramic Hydroxyapatite columns were used to isolated high acidic (activity) tg-FIX. These two chromatography enriched active tg-FIX subpopulation, resulting in a specific activity increased more than 10 fold (Table 1). Finally, the property of γ -carboxylglutamic acid residue conducting a size compaction in the presence of

calcium ions was utilized to further separated active tg-FIX subpopulation from the non-active tg-FIX subpopulations by HPLC-SEC; and the activated/degraded tg-FIX subpopulation was also eliminated caused by the molecular weight difference.

We noticed the amount of activated and degraded tg-FIX decreased about 9% (mass) by adding the protease inhibitors. By co-expressing tg-furin, which processes excessive pro-peptide in transgenic milk [paper in draft], no extra cleavage step needed and EDTA and PIs can be added right after milking. This will further improve the yield of active tg-FIX by decreasing activation and degradation of tg-FIX.

The overall yield of active tg-FIX was approximated 3% of whole tg-FIX population in the transgenic swine milk by using the purification protocol (Table 3). The final product, active tg-FIX, had about 200 U/mg FIX specific activity without any detected activated tg-FIX (tg-FIXa). Assuming average tg-FIX expression rate in pig was 2.5 mg/ml, and milk production was 400 liters per pig and per year, approximately 40 pigs could produce enough FIX concentrates for US consumption (250 million units) and 160 pigs was enough for the worldwide (1 billion units). We are now working on the project of introducing the vitamin K 2,3-epoxide reductase enzyme (VORK), which produces the cofactor for γ -carboxylase, gene and co-expressing with tg-FIX to accelerate the formation of γ -carboxylglutamate (Gla) that is relative pro-coagulant activity(40). The active tg-FIX subpopulation could be further increased.

REFERENCES

1. WFH. (2013) WFH Report on the Annual Global Survey 2012 Available at <http://www1.wfh.org/publications/files/pdf-1574.pdf>
2. Panicker, J., Warrier, I., Thomas, R., and Lusher, J. M. (2003) The overall effectiveness of prophylaxis in severe haemophilia. *Haemophilia : the official journal of the World Federation of Hemophilia* **9**, 272-278
3. Franchini, M., Frattini, F., Crestani, S., Sissa, C., and Bonfanti, C. (2013) Treatment of hemophilia B: focus on recombinant factor IX. *Biologics : targets & therapy* **7**, 33-38
4. Stonebraker, J. S., Bolton-Maggs, P. H. B., Brooker, M., Farrugia, A., and Srivastava, A. (2011) A study of reported factor IX use around the world. *Haemophilia* **17**, 446-455
5. Gater, A., Thomson, T. A., and Strandberg-Larsen, M. (2011) Haemophilia B: impact on patients and economic burden of disease. *Thromb Haemost* **106**, 398-404
6. Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W., and Kurachi, K. (1985) Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* **24**, 3736-3750
7. Kurachi, K., and Kurachi, S. (1995) Regulatory mechanism of the factor IX gene. *Thromb Haemost* **73**, 333-339
8. Burnier, J. P., Borowski, M., Furie, B. C., and Furie, B. (1981) Gamma-carboxyglutamic acid. *Mol Cell Biochem* **39**, 191-207
9. Morris, D. P., Stevens, R. D., Wright, D. J., and Stafford, D. W. (1995) Processive post-translational modification. Vitamin K-dependent carboxylation of a peptide substrate. *J Biol Chem* **270**, 30491-30498
10. Bravo, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) Accurate and efficient cleavage of the human insulin proreceptor by the human proprotein-processing protease furin. Characterization and kinetic parameters using the purified, secreted soluble protease expressed by a recombinant baculovirus. *J Biol Chem* **269**, 25830-25837
11. Steiner, D. F. (1998) The proprotein convertases. *Curr Opin Chem Biol* **2**, 31-39
12. Nishimura, H., Kawabata, S., Kisiel, W., Hase, S., Ikenaka, T., Takao, T., Shimonishi, Y., and Iwanaga, S. (1989) Identification of a disaccharide (Xyl-Glc) and a trisaccharide (Xyl₂-Glc) O-glycosidically linked to a serine residue in the first epidermal growth factor-like domain of human factors VII and IX and protein Z and bovine protein Z. *J Biol Chem* **264**, 20320-20325
13. Nishimura, H., Takao, T., Hase, S., Shimonishi, Y., and Iwanaga, S. (1992) Human factor IX has a tetrasaccharide O-glycosidically linked to serine 61 through the fucose residue. *J Biol Chem* **267**, 17520-17525
14. Harris, R. J., van Halbeek, H., Glushka, J., Basa, L. J., Ling, V. T., Smith, K. J., and Spellman, M. W. (1993) Identification and structural analysis of the tetrasaccharide NeuAc alpha(2-->6)Gal beta(1-->4)GlcNAc beta(1-->3)Fuc alpha 1-->O-linked to serine 61 of human factor IX. *Biochemistry* **32**, 6539-6547
15. Agarwala, K. L., Kawabata, S., Takao, T., Murata, H., Shimonishi, Y., Nishimura, H., and Iwanaga, S. (1994) Activation peptide of human factor IX has

- oligosaccharides O-glycosidically linked to threonine residues at 159 and 169. *Biochemistry* **33**, 5167-5171
16. Fernlund, P., and Stenflo, J. (1983) Beta-hydroxyaspartic acid in vitamin K-dependent proteins. *J Biol Chem* **258**, 12509-12512
 17. Kaufman, R. J. (1998) Post-translational modifications required for coagulation factor secretion and function. *Thromb Haemost* **79**, 1068-1079
 18. Atoda, H., Yokota, E., and Morita, T. (2006) Characterization of a monoclonal antibody B1 that recognizes phosphorylated Ser-158 in the activation peptide region of human coagulation factor IX. *J Biol Chem* **281**, 9314-9320
 19. White, G. C., 2nd, Beebe, A., and Nielsen, B. (1997) Recombinant factor IX. *Thromb Haemost* **78**, 261-265
 20. Arruda, V. R., Hagstrom, J. N., Deitch, J., Heiman-Patterson, T., Camire, R. M., Chu, K., Fields, P. A., Herzog, R. W., Couto, L. B., Larson, P. J., and High, K. A. (2001) Posttranslational modifications of recombinant myotube-synthesized human factor IX. *Blood* **97**, 130-138
 21. Bond, M., Jankowski, M., Patel, H., Karnik, S., Strang, A., Xu, B., Rouse, J., Koza, S., Letwin, B., Steckert, J., Amphlett, G., and Scoble, H. (1998) Biochemical characterization of recombinant factor IX. *Semin Hematol* **35**, 11-17
 22. Van Cott, K. E., Monahan, P. E., Nichols, T. C., and Velander, W. H. (2004) Haemophilic factors produced by transgenic livestock: abundance that can enable alternative therapies worldwide. *Haemophilia* **10 Suppl 4**, 70-76
 23. Lubon, H., Paleyanda, R. K., Velander, W. H., and Drohan, W. N. (1996) Blood proteins from transgenic animal bioreactors. *Transfus Med Rev* **10**, 131-143
 24. Wang, Y., Zhao, S., Bai, L., Fan, J., and Liu, E. (2013) Expression systems and species used for transgenic animal bioreactors. *BioMed research international* **2013**, 580463
 25. Clark, A. J., Bessos, H., Bishop, J. O., Brown, P., Harris, S., Lathe, R., Mcclenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B. A., and Wilmut, I. (1989) Expression of Human Anti-Hemophilic Factor-Ix in the Milk of Transgenic Sheep. *Bio-Technol* **7**, 487-492
 26. Zhang, K., Lu, D., Xue, J., Huang, Y., and Huang, S. (1997) Construction of mammary gland-specific expression vectors for human clotting factor IX and its secretory expression in goat milk. *Chinese journal of biotechnology* **13**, 271-276
 27. Lindsay, M., Gil, G. C., Cadiz, A., Velander, W. H., Zhang, C., and Van Cott, K. E. (2004) Purification of recombinant DNA-derived factor IX produced in transgenic pig milk and fractionation of active and inactive subpopulations. *J Chromatogr A* **1026**, 149-157
 28. Richt, J. A., Kasinathan, P., Hamir, A. N., Castilla, J., Sathiyaseelan, T., Vargas, F., Sathiyaseelan, J., Wu, H., Matsushita, H., Koster, J., Kato, S., Ishida, I., Soto, C., Robl, J. M., and Kuroiwa, Y. (2007) Production of cattle lacking prion protein. *Nature biotechnology* **25**, 132-138
 29. Thiel, K. A. (2004) Biomanufacturing, from bust to boom...to bubble? *Nature biotechnology* **22**, 1365-1372
 30. Detmers, F., Hermans, P., Jiao, J., McCue, J. T. (2010) Novel Affinity Ligands Provide for Highly Selective Primary Capture. *BioProcess Internatinal* **8**, 50-54

31. Rasband, W. S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2012.
32. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671-675
33. Gui, T., Lin, H. F., Jin, D. Y., Hoffman, M., Straight, D. L., Roberts, H. R., and Stafford, D. W. (2002) Circulating and binding characteristics of wild-type factor IX and certain Gla domain mutants in vivo. *Blood* **100**, 153-158
34. Jagadeeswaran, P., and Sheehan, J. P. (1999) Analysis of blood coagulation in the zebrafish. *Blood Cells, Molecules, and Diseases* **25**, 239-249
35. Monroe, D. M., Noyes, C. M., Straight, D. L., Roberts, H. R., and Griffith, M. J. (1985) Activation of normal and abnormal human factor IX with trypsin. *Archives of biochemistry and biophysics* **238**, 490-496
36. Paleyanda, R. K., Velander, W. H., Lee, T. K., Scandella, D. H., Gwazdauskas, F. C., Knight, J. W., Hoyer, L. W., Drohan, W. N., and Lubon, H. (1997) Transgenic pigs produce functional human factor VIII in milk. *Nature biotechnology* **15**, 971-975
37. Velander, W. H., Johnson, J. L., Page, R. L., Russell, C. G., Subramanian, A., Wilkins, T. D., Gwazdauskas, F. C., Pittius, C., and Drohan, W. N. (1992) High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc Natl Acad Sci U S A* **89**, 12003-12007
38. Lee, H. G., Lee, H. C., Kim, S. W., Lee, P., Chung, H. J., Lee, Y. K., Han, J. H., Hwang, I. S., Yoo, J. I., Kim, Y. K., Kim, H. T., Lee, H. T., Chang, W. K., and Park, J. K. (2009) Production of recombinant human von Willebrand factor in the milk of transgenic pigs. *The Journal of reproduction and development* **55**, 484-490
39. Gil, G. C., Velander, W. H., and Van Cott, K. E. (2008) Analysis of the N-glycans of recombinant human Factor IX purified from transgenic pig milk. *Glycobiology* **18**, 526-539
40. Wajih, N., Hutson, S. M., Owen, J., and Wallin, R. (2005) Increased production of functional recombinant human clotting factor IX by baby hamster kidney cells engineered to overexpress VKORC1, the vitamin K 2,3-epoxide-reducing enzyme of the vitamin K cycle. *J Biol Chem* **280**, 31603-31607

CHAPTER 5

SUMMARY AND FUTURE WORK

SUMMARY

The success in expressing recombinant human FIX (tg-FIX) with approximately 2-3 g/L and 10-20% bio-active in the milk (1) makes this mammary gland of transgenic pig a promising bioreactor in producing recombinant human FIX with full pro-coagulate activity. The goal of this project was to increase the yield of the bio-active tg-FIX, and this was fulfilled by the following specific aims:

Specific Aim#1: Characterize the tg-FIX and its degraded species in the milk sample.

And investigate the predominant milk-borne protease, plasmin, in degrading the tg-FIX.

Specific Aim#2: Bioengineer pig mammary gland by introducing truncated human furin gene co-expressing with tg-FIX. Characterize the recombinant truncated human furin (r-furin) in processing the excessive pro-peptide and convert non-active tg-proFIX to active tg-FIX both *in vivo* and *in vitro*.

Specific Aim#3: Establish scaled-up purification protocol, using the more efficient, immune-affinity chromatography FIX select column to capture whole tg-FIX population from transgenic milk; then enrich high acidic tg-FIX by anion exchange chromatography; finally separate the active tg-FIX subpopulation from the inactive and activated tg-FIX subpopulation.

We noticed about 50% of the high extent γ -carboxylated tg-FIX was activated or degraded in the purified high acidic tg-FIX. By investigating the degraded species, plasmin, which is the predominant protease in milk, was involved the degrading and

facilitate activating the tg-FIX in the milk. The extent of degradation/activation of tg-FIX could be decreased by introducing the protease inhibitors, by introducing the ϵ -aminocaproic acid (EACA), which is the specific inhibitor of plasmin, and benzamidine during the storage of milk. The percentage of degraded/activated tg-FIX dropped from 43% to 34%. However the degradation and activation before collecting milk could not be reversed. We noticed the tg-FIX having high extent Gla, which conducted the conformation change in Gla domain in presence of calcium ion, was prevented light chain being cleaved by plasmin. However, cleaved at C-terminal of catalytic domain and deactivated the tg-FIX happened not dependent on its Gla content and in presence of calcium ion or in chelated environment. The plasmin also performed cleavage at Arg¹⁴⁵, making FIX α (intermediate of activated FIX), facilitated the activation of tg-FIX, which only needed one more cleavage at Arg¹⁸⁰. In order to increase the yield of active tg-FIX, the action of the protease in the milk need to minimize.

In the tg-FIX expressed only transgenic pig, we noticed approximately 10-30% pro-peptide attached tg-FIX, which was not processed by endogenous porcine furin in the mammary epithelium during the tg-FIX synthesis. And this unprocessed pro-tgFIX, which interferes conformational changes in Gla domain causing no pro-coagulation activity, could not be separated by current purification methods. This causes the decreasing in FIX specific activity of the purified tg-FIX sample; in addition, the pro-tgFIX, which has high extent Gla, could be waste if we left it unprocessed. By introducing the truncated human furin cDNA and co-expressing with the tg-FIX, this tg-furin, with furin specific activity, helped the endogenous porcine furin in processing over-expressed nascent tg-FIX. In addition, without the transmembrane domain, this tg-

furin secreted into the milk, and approved to process pro-peptide *in vitro* also. No pro-tgFIX was detected with co-expressing this tg-furin. More important, no evidence showed this co-expressed tg-furin decreasing the percentage of the active tg-FIX, which indicated the over-expressed tg-furin did not interfere the formation of gamma-carboxylation in tg-FIX in ER.

The scaled-up purification protocol was established with modifying the previous lab-scale one. By introducing the new camelid IgG based immunoaffinity chromatography, the capture of whole tg-FIX population is more efficient than the current heparin based affinity chromatography. The anion exchange chromatography further enriched the high acidic tg-FIX subpopulation, which is highly related to its extent of Gla. And the HPLC-SEC not only separated the active tg-FIX from non-active (low extent of Gla) tg-FIX, and also the activated, also high extent Gla, tg-FIX by their size difference in presence of calcium ion. The final tg-FIX showed similar specific activity to the plasma-derived and recombinant (CHO) FIX.

FUTURE WORK

Approximately half of the high acidic tg-FIX subpopulation were degraded or activated in the milk sample and during the processing. The degradation and activation after tg-FIX secreting into the milk could not be reversed. The plasmin specific inhibitor, α_2 -antiplasmin (A2AP), could be the candidate in inhibiting the plasmin activity in the milk(2). The efficiency of co-expressing this serine protease inhibitor with the tg-FIX needs to be investigated. Also, the precaution needs to be taken, since both tg-FIX and furin are serine protease, the co-expressed A2AP might inhibit their activity. In addition,

since the A2AP involves in inhibiting plasmin activity in blood, the current purification protocol and the final purified tg-FIX need to be investigated to remove all A2AP.

The current expression level of recombinant human FIX in milk of transgenic pig is about 2-3 g/L, however, only 10-20% high acidic/carboxylated FIX population could be enhanced. It is believed that incomplete-carboxylation occurs when an excess of newly synthesized precursors of vitamin K-dependent proteins appears in the ER and exceeds the capacity of the cell's-carboxylation system to fully modify all of the precursors (3,4). The under-carboxylated FIX is less active, prone to be degraded, and cleared more rapidly from the bloodstream than fully carboxylated FIX (5). The reduced form of vitamin K, vitamin K hydroquinone (KH₂), is the cofactor as carboxylase in the reaction of γ -carboxylation(6). This KH₂ is recycle between its oxidized form by the enzyme, vitamin K epoxide reductase (VKOR)(6). So, this VKOR maintains the level of the KH₂ and also accelerate the reaction of carboxylase(7,8), and the truncated recombinant human VKOR, an 18-kDa ER membrane protein VKORC1(9), was expressed and proven in increasing production of functional FIX in the CHO cells(10). With the success in bioengineering of the mammary gland of pig in processing excessive pro-peptide of FIX by co-expressing the truncated recombinant human furin, introducing this VKORC1 could increase the yield of fully carboxylated FIX.

At the meanwhile, the *in vivo* effects of our recombinant human FIX, expressed and purified as in the previous study, should be investigated in pharmacokinetics by using animal models. The wild type mice (as control), hemophilia mice, including R333Q mice(11) and FIX knockout (FIXKO) mice (12), and hemophilia dog(13) will be administrated intravenously using purified active tg-FIX in multiple doses and the FIX

antigen and activity level will be monitored by the ELISA and aPTT, respectively, at multiple time points after administration. Oral administration of coagulation therapy will obviate the invasiveness, discomfort, potential for opportunistic infection, and complications of storage and supplies that accompany with current intravenous administration. With the progress in producing recombinant human FIX economically in mammary gland of transgenic pig, the cost of the FIX concentrates would drop and make this oral administration affordable. The safety and efficiency of oral administration of this recombinant human FIX should also be investigated by using the animal models that were used in intravenous administration.

REFERENCES

1. Lindsay, M., Gil, G. C., Cadiz, A., Velander, W. H., Zhang, C., and Van Cott, K. E. (2004) Purification of recombinant DNA-derived factor IX produced in transgenic pig milk and fractionation of active and inactive subpopulations. *J Chromatogr A* **1026**, 149-157
2. Giles, A. R., Nesheim, M. E., Herring, S. W., Hoogendoorn, H., Stump, D. C., and Heldebrant, C. M. (1990) The fibrinolytic potential of the normal primate following the generation of thrombin in vivo. *Thromb Haemost* **63**, 476-481
3. Berkner, K. L. (1993) Expression of recombinant vitamin K-dependent proteins in mammalian cells: factors IX and VII. *Methods Enzymol* **222**, 450-477
4. Kaufman, R. J., Wasley, L. C., Furie, B. C., Furie, B., and Shoemaker, C. B. (1986) Expression, purification, and characterization of recombinant gamma-carboxylated factor IX synthesized in Chinese hamster ovary cells. *J Biol Chem* **261**, 9622-9628
5. Gui, T., Lin, H. F., Jin, D. Y., Hoffman, M., Straight, D. L., Roberts, H. R., and Stafford, D. W. (2002) Circulating and binding characteristics of wild-type factor IX and certain Gla domain mutants in vivo. *Blood* **100**, 153-158
6. Furie, B., and Furie, B. C. (1991) Molecular basis of gamma-carboxylation. Role of the propeptide in the vitamin K-dependent proteins. *Ann N Y Acad Sci* **614**, 1-10
7. Suttie, J. W. (1993) Synthesis of vitamin K-dependent proteins. *FASEB J* **7**, 445-452
8. Wallin, R., Sane, D. C., and Hutson, S. M. (2002) Vitamin K 2,3-epoxide reductase and the vitamin K-dependent gamma-carboxylation system. *Thromb Res* **108**, 221-226

9. Cain, D., Hutson, S. M., and Wallin, R. (1997) Assembly of the warfarin-sensitive vitamin K 2,3-epoxide reductase enzyme complex in the endoplasmic reticulum membrane. *J Biol Chem* **272**, 29068-29075
10. Wajih, N., Hutson, S. M., Owen, J., and Wallin, R. (2005) Increased production of functional recombinant human clotting factor IX by baby hamster kidney cells engineered to overexpress VKORC1, the vitamin K 2,3-epoxide-reducing enzyme of the vitamin K cycle. *J Biol Chem* **280**, 31603-31607
11. Bajaj, S. P., Schmidt, A. E., Mathur, A., Padmanabhan, K., Zhong, D., Mastro, M., and Fay, P. J. (2001) Factor IXa:factor VIIIa interaction. helix 330-338 of factor IXa interacts with residues 558-565 and spatially adjacent regions of the $\alpha 2$ subunit of factor VIIIa. *J Biol Chem* **276**, 16302-16309
12. Lin, H. F., Maeda, N., Smithies, O., Straight, D. L., and Stafford, D. W. (1997) A coagulation factor IX-deficient mouse model for human hemophilia B. *Blood* **90**, 3962-3966
13. Nichols, T. C., Franck, H. W., Franck, C. T., De Friess, N., Raymer, R. A., and Merricks, E. P. (2012) Sensitivity of whole blood clotting time and activated partial thromboplastin time for factor IX: relevance to gene therapy and determination of post-transfusion elimination time of canine factor IX in hemophilia B dogs. *J Thromb Haemost* **10**, 474-476