Production of Recombinant Human Von Willebrand Factor in the Milk of Transgenic Pigs

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Abstract. Von Willebrand factor (vWF), a large multimeric glycoprotein present in blood plasma, is a blood protein of the coagulation system. It is defective in von Willebrand disease and is involved in a large number of other diseases, including thrombotic thrombocytopenic purpura-hemolytic uremic syndrome and heyde’s syndrome. We have developed a line of transgenic swine harboring recombinant human von Willebrand factor (rhvWF) cDNA through microinjection of fertilized one-cell pig zygotes. Expression of rhvWF in the mammary gland and secretion of rhvWF into the milk of the transgenic swine were confirmed by immunohistochemical and western blot analyses, respectively, and rhvWF proteins were detected in milk from all lactating founder females at concentrations that were 28- to 56-folds greater than that in circulating human plasma. The amino acid sequence of rhvWF protein in the transgenic pig milk matched that of vWF produced from human blood plasma. This study provides evidence that production of rhvWF from transgenic pig milk is a potentially valuable technology and can be used as a cost-effective alternative in clinical applications.

Key words: Mammary gland, Microinjection, Transgenic pig, Von Willebrand factor

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On Willebrand factor (vWF or factor VIII-related antigen)—the deficiency of which causes von Willebrand disease (vWD), the most common inherited bleeding disorder—is constitutively produced in the endothelium, megakaryocytes and subendothelial connective tissue as a large multimeric glycoprotein circulating in blood plasma [1, 2]. It is also involved in a large number of other diseases, including thrombotic thrombocytopenic purpura-hemolytic uremic syndrome and heyde’s syndrome [3, 4], mediates platelet adhesion to sites of vascular injury by binding to specific platelet membrane glycoproteins in a closely regulated manner and functions as a carrier protein for blood clotting factor VIII (FVIII) [5]. In the circulation, vWF mainly functions by binding to other proteins, particularly FVIII, during the process of coagulation, and the absence of this interaction causes rapid degradation of vWF. In regard to the apparent role of vWF in high shear stress, vWD deficiency leads to a tendency to bleed, most apparent in tissues with high blood flow in narrow vessels [2]. The basic vWF monomer is a 2,050 amino acid protein and is assembled via identical 250-kDa subunits into disulfide-linked multimers that may vary between 2 and >20; thus, the molecular mass of the heterogeneously sized vWF protein circulating in plasma ranges from $5 \times 10^5$ Da to more than $5 \times 10^6$ Da [1, 2].

The development of a method for production of a highly purified protein on an economically large scale is one of the targets of modern animal biotechnology research. Gordon et al. were the first to attempt to produce recombinant proteins in the milk of transgenic (TG) mice [6]; since then, modification of phenotypic properties became possible [7]. Of the transgenic technologies employing domestic animals that have been developed over the last decade, one of the most popular applications has been the generation of animal bioreactors for production of therapeutic proteins [8–12]. The pig is an attractive animal for this kind of application due to its fecundity and physiology that closely resembles that of humans [13]. Mass production of therapeutic proteins in the milk of TG pigs is a cost-effective alternative to production by cell culture or fermentation [14]. There have been several reports on use of the pig as an animal bioreactor for recombinant proteins such as human FVIII [15], human hemoglobin [16], human protein C [17] and human erythropoietin [9]. Despite the usefulness of this technique for analysis of protein functions and development of model animals for studying diseases, there have been only a few reports on large-scale production of TG pigs expressing human proteins. Furthermore, generation of a TG pig harboring the human vWF gene has been unsuccessful thus far.

Here, we report generation of TG pigs producing active rhvWF in their milk. We have established a practical system by which large quantities of rhvWF can be produced in the milk of sows throughout the lactation period, and we tested the binding activity of rhvWF secreted into the milk of transgenic pigs.

Materials and Methods

Production of bovine α-S1 casein-hvWF transgenic pigs

The expression vector pBlue-bCSN1S1-hvWF (18,257 bp) was cloned with 6,474 bp of the bovine α-S1 casein (bCSN1S1) pro-
moter [18], 8,442 bp of human vWF (hvWF) cDNA and 294-bp of bovine growth hormone (bGH) polyadenylation signal sequences with pBluescript as the backbone. Landrace (Sus scrofa domestica) gilts were injected with PG 600 (Intervet International BV, Boxmeer, The Netherlands) at 200–230 days of age. Gilts that responded to the injection by exhibiting standing estrus continued to be observed. They were administered 1500 IU of PMSG (Intervet International BV, Boxmeer, The Netherlands) by subcutaneous injection 16 days after standing estrus and then 750 IU of hCG (Intervet International BV, Boxmeer, The Netherlands) by intramuscular injection 72 h after the PMSG injection. Donors were bred by natural mating at 24 h after the hCG injection. Embryos were recovered surgically at 30–32 h after insemination. The reproductive tracts of donor gilts were exposed by midventral laparotomy under general anesthesia. Ova were collected from the oviducts by flushing with 20 ml of sterile Dulbecco’s phosphate-buffered saline (Gibco BRL). The ova were centrifuged at 15,000 g for 5 to 10 min to visualize the pronuclei, and the pronucleus of one-celled ova were microinjected with TE (Sigma, Chemical, St. Louis, MO, USA) solution containing 4 ng/μl of a 15.21-kb Not I/Sal I restriction fragment of the PBlue-bCSN1S1-hvWF vector carrying the bCSN1S1 promoter, hvWF cDNA and bGH poly-A signal sequences. The prepared DNA construct was microinjected using a micromanipulator into the 1-cell stage embryos. The microinjected 1-cell embryos were surgically inserted into the oviduct-synchronized pigs [19]. The estrous cycles of all the recipients were synchronized by the same method as the donors. At 2 days after hCG administration, an average of approximately 26 of the injected eggs were transferred into each recipient’s oviduct using a micromanipulator into the 1-cell stage embryos. The microinjected 1-cell embryos were surgically inserted into the oviduct-synchronized pigs [19]. The estrous cycles of all the recipients were synchronized by the same method as the donors. At 2 days after hCG administration, an average of approximately 26 of the injected eggs were transferred into each recipient’s oviduct using a fine capillary tube with a small volume of NCsu23 medium including 0.1% BSA. The founders harboring the rhvWF gene were identified by performing a polymerase chain reaction (PCR) and Southern blotting. Male founders were used to produce first-generation TG piglets by natural mating with wild-type swine, while female founders were naturally mated with wild-type boars. The Animal Care and Concern Committee of the National Livestock Research Institute approved the animal protocols used in this study. TG piglets were identified by performing PCR using genomic DNA purified from the tail tips of newborn piglets. We used 2 sets of PCR primers to produce 2 individual amplification products comprising 605 (forward 5'-AAAACGTGCCAGAACTATGAC-3' and reverse 5'-TCCCAGCTTCTTATTTTGATG-3') and 1,085 (forward 5'-TCCCAGCTTCTTATTTTGATG-3' and reverse 5'-TGGAGTGAAATGTGAAGATGTG-3') nucleotides (nts).

**Southern hybridization**

Five micrograms of blood DNA was digested with Pst I at 37 C overnight. Samples (F0–F3) were separated 1.0% agarose gel and blotted overnight onto a nylon membrane. A 1.8-kb Pst I fragment of vWF cDNA was DIG-labeled and hybridized to blots. The procedure was carried out as described in the included instructions of the DIG DNA labeling and detection kit (Roche, Indianapolis, IN, USA). A 605-nt amplification fragment was produced by using PCR primers and was used as a hybridization probe in southern blotting. To determine the copy numbers of the transgene integrated into the genome of a transgenic pig (F0–F3), 5 μg of purified genomic DNA of the transgenic pig (F0–F3) and the bCSN1S1-hvWF-bGH fragment, which was diluted to make a series of copy number controls (1–100 copies per genome), were digested by Pst I and probed with the DIG-labeled probe. The transgene copy number was determined by comparison of the hybridization signal with the copy number controls using the background subtraction method.

**RT-PCR analysis with mammary gland tissue**

Total RNA was isolated from fresh mammary gland tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Following total RNA extraction, cDNA was synthesized from 1 μg (20 μl reaction) of total RNA using a 1st Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics, Manheim, Germany). PCR was performed using a gene specific primer (vWF-605 bp, porcine GAPDH) and recombinant Taq DNA polymerase (TaKaRa, Tokyo, Japan).

**In vitro culture of mammary gland tissue**

Mammary gland tissue was surgically obtained from Landrace gilts during the lactation period (5 weeks after parturition) by excisional biopsy and cut with a blade. The tissue was washed 5 times with 1X D-PBS (Dulbecco’s phosphate-buffered saline, Invitrogen) and once with growth media-DMEM (Dulbecco’s modified Eagle’s medium)/F-12 (Invitrogen) supplemented with 10% FBS (heat-inactivated fetal bovine serum, Invitrogen), 5 μg/ml insulin (Sigma), 10 ng/ml EGF (mouse epidermal growth factor, Sigma), 8442 bp of human vWF (hvWF) cDNA and 294-bp of bovine growth hormone (bGH) polyadenylation signal sequences. The tissue was cultured for 10 days in a 75-cm² plastic tissue culture flask and subcultured using an 8-μm cell strainer (Sigma; ratio of 1:2 at 3 day intervals). Mammary gland tissues were grown in growth media in an incubator at 38.5 C in a humidified atmosphere with 5% CO₂.

**Immunohistochemical analysis of rhvWF in the mammary gland**

Frozen tissue samples of wild-type and hvWF-expressing swine were cut into 5-μm slices, mounted on poly-(L-lysine)-coated slides and washed 10 times in phosphate-buffered saline (PBS). For antigen retrieval, the sections were fixed in acetone solution for 10 min and probed with the DIG-labeled probe. The transgene copy number was determined by comparison of the hybridization signal with the copy number controls using the background subtraction method.

**Western blot analysis of rhvWF in milk**

Milk fat was removed from the sow whole milk by centrifuga-
tion at approximately $8,000 \times g$ for 15 min at 4°C. A total of 30 $\mu l$ of skimmed milk was diluted 16- to 64-fold, mixed with 10 $\mu l$ of SDS-PAGE sample buffer, resolved by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 6% gel and finally transferred onto nitrocellulose (NC) membranes (Amersham Biosciences, USA). The membranes were incubated for 1 h in blocking buffer (20 mM Tris-HCl-buffered saline containing 5% nonfat milk powder and 0.1% Tween-20) at room temperature (RT) and then probed with an rabbit anti-human vWF polyclonal antibody, A0082 (1:1,000), in blocking buffer overnight at 4°C. The blots were then incubated with secondary anti-rabbit IgG-HRP conjugates (1:10,000) and developed by using the enhanced chemiluminescence (ECL) method.

Quantification of the rhvWF protein in milk

Skimmed milk was diluted with 4 volumes of PBS (pH 7.4) and by centrifuged (13,000 $\times g$ at 4°C for 20 min), and the whey was then removed from the fat layer and the insoluble precipitate. The milk samples were diluted in the range of 4- to 262,144-fold. An enzyme-linked immunosorbent assay (ELISA) was performed to determine the levels of human vWF in the milk of the TG pigs by using an in-house kit [20]. We used 2 rabbit anti-human vWF antibodies conjugated with HRP as the capture (A0082) and detector antibodies (P0226, Dako, Denmark). The assay was performed according to the instructions provided in the supplier’s manual, and normal human plasma (Dade Behring, Germany) was used as a vWF standard.

Milk processing and chromatography

A total of 73 ml of skimmed milk was collected and mixed with 0.73 ml of 500 mM ethylenediaminetetraacetic acid (EDTA; to a final concentration of 5 mM EDTA). Cryoprecipitation involved 1 cycle of freezing (−70°C) and thawing (4°C), centrifugation of the solution (8,000 $\times g$ at 4°C for 15 min) and then collection of the supernatant for further processing. Following skimming and cryoprecipitation, saturated ammonium sulfate solution (4.01 M) was added to the supernatant to a final concentration of 40%. The saturated ammonium sulfate solution was added slowly to the samples with gentle stirring under ice conditions in order to avoid creation of localized regions with very high concentrations of ammonium sulfate. The samples were then centrifuged (10,000 $\times g$ at 4°C for 15 min), and the pellets were suspended in 50 ml heparin column equilibration buffer and dialyzed overnight at 4°C against 20 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. The dialyzed samples were then applied to protein A-agarose columns that had previously been equilibrated with 20 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. The flow-through fractions were collected and directly loaded onto SP sepharose 4B fast-flow resin columns (Amersham Pharmacia, USA) that had previously been equilibrated with 20 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. After washing with 20 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, the proteins were eluted with a solution containing 20 mM Tris-HCl (pH 7.4) and 1 M NaCl. The eluates obtained in each step were separated by SDS-PAGE and subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis [21].

MS characterization

The purified protein was excised from the gel and digested with trypsin, as described by Jensen et al. [21]. The resulting peptide mixture was analyzed by MALDI-MS and ESI-Q-TOF MS/MS using a nano-ESI on a Q-TOF mass spectrometer (Micromass, Manchester, UK). To identify the protein, all MS/MS spectra recorded on tryptic peptides derived from gel and amino acid sequences deduced using a peptide de-novo sequencing program, PepSeq, were searched against protein sequences from the NCBInr and EST databases using the MASCOT search program (www.matrixscience.com) and BLAST.

Analysis of the multimeric structure of rhvWF and FVIII-rhvWF interactions

The vWF multimer was analyzed by performing horizontal SDS-agarose gel electrophoresis on a 1.5% gel, and the vWF multimers were visualized by immunoenzymatic staining [22]. The anti-human vWF antibody P0226 and alkaline phosphatase (AP)-conjugated affinity-purified goat anti-rabbit IgG (Axell, USA) were used as the primary and secondary antibodies, respectively, and were developed by electrochemiluminescence (ECL; Amersham Life Science, USA). The ability of purified vWF to bind to FVIII was assessed by performing a modified ELISA to detect the FVIII-vWF complex [23, 24]. A sheep anti-human FVIII polyclonal antibody (CL20035K; Cedarlane, Canada) was diluted with carbonate buffer (pH 9.6) and coated over a microplate at 4°C overnight. Each well was blocked with 3% bovine serum albumin (BSA) in PBS (pH 7.2) for 1 h at RT. Following 4 washings with PBS (pH 7.2) containing 0.1% Tween-20 (washing buffer), various amounts of purified FVIII, ranging from 0.125 to 8 IU/ml, were added, and the plates were incubated for 2 h. Following incubation and washing, 3 different concentrations of the vWF standard in FVIII-deficient plasma (Dade Behring, Germany) or human vWF purified from the TG-pig milk were added, and the plates were then incubated at RT for 2 h. Subsequently, an antibody, P0026, was added, and the plates were incubated for 1 h. The plates were washed 3 times with washing buffer, o-phenylenediamine dihydrochloride was added to each well as a substrate and the plates were allowed to stand for 15 min for color development. The color reaction was arrested by adding 50 $\mu l$ of 2.5 M H$_2$SO$_4$ to the wells. The plates were read at a wavelength of 490 nm by using a microplate reader. The optical density was plotted against the FVIII concen-
Production of rhvWF in the milk of TG pigs

Concentrations at fixed concentrations (50, 100 and 200 ng/ml) of vWF obtained from normal human plasma and the milk of TG pigs.

**Results**

**Production of bCSN1S1-hvWF transgenic pigs**

A linearized pBlue-bCSN1S1-hvWF expression vector was microinjected into the pronuclei of 488 embryos. A total of 434 microinjected single-cell embryos were transferred into 18 recipients. As shown in Table 1, pregnancy was successfully maintained in some recipients. Five piglets delivered by 4 recipients (from among 123 embryos) were screened as transgenic piglets by performing genomic PCR (Fig. 1B). The integrity of the transgene DNA and its copy number were determined by southern blot analysis with *Pst I*, which makes a double cut at hvWF cDNA, resulting in a generation of a 1.8-kb fragment. Transgenic founder (F0) had approximately 20 copies of the transgene, and the copy number of the transgene in its offspring (F1, F2 and F3) showed a similar trend (Fig. 1C).

The average rate of TG pig production was 19.23%, including 2 postnatal deaths (Table 1). F1 offsprings were produced by natural mating between the founders and wild-type pigs; 4 pigs gave birth to 37 piglets, including 16 TG piglets (of which 3 were deceased). The male to female ratio for both the total progeny and TG offsprings was approximately 1:1 in the F1 generation.

**Expression of rhvWF in the mammary gland**

The rhvWF concentration in the TG pig milk was determined by measuring its concentration in 1 ml of skimmed milk from the TG sows by performing ELISA. During the 30-day lactation period, the mean rhvWF expression level for the lactating sows (31–278, founder) was 280 μg/ml (data not shown). SDS-PAGE on a 6% gel followed by western blotting revealed the presence of the rhvWF protein in the whole milk of the TG sows and its molecular weight. Western blot analysis performed using a rabbit anti-human vWF polyclonal antibody known to strongly cross-react with porcine vWF (pvWF) did not reveal the presence of pvWF in the control milk samples, and rhvWF was more highly expressed in the TG pig milk than in the normal human plasma at all examined diluted concentrations (Fig. 2A). The expression of rhvWF was also confirmed by performing RT-PCR using hvWF-specific primers (Fig. 2B), and rhvWF was detected in the mammary glands of the wild-type and TG swine by using rabbit anti-human-vWF/HRP polyclonal antibodies (Fig. 3).

**Purification and analysis of rhvWF in milk**

The purification steps, including ion-exchange chromatography, were used to isolate human vWF from the skimmed TG pig milk. After SP (ion-exchange chromatography) elutes, rhvWF density according to the dilution concentration was detected by immunoblotting and did not disappear the rhvWF protein (data not shown). Western blotting was performed to confirm the presence of rhvWF in each eluate at the end of each purification step. It appears that bands of more than 180 kDa corresponded to vWF multimers and that approximately 90% vWF could be eluted by heparin chromatography following cryoprecipitation and ammonium sulfate precipitation. The sow skimmed milks of the non-TG and TG pigs separated by SDS-PAGE are shown in Fig. 4. The band of putative rhvWF, band 3, was not detected in the non-TG skimmed milk. The eluates obtained after ion-exchange chromatography were sub-

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**Table 1. Analysis of transgenic gene transfer in progenies**

<table>
<thead>
<tr>
<th>Classes</th>
<th>No. of recipients</th>
<th>No. of piglets</th>
<th>Transgenic pigs</th>
<th>Gene transfer rate (%)</th>
<th>Method of production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sows</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>F0</td>
<td>18</td>
<td>12</td>
<td>14</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>F1</td>
<td>4</td>
<td>18 a</td>
<td>19 b</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

A total of 488 microinjected embryos were transferred into 18 recipients. Twenty-six piglets were delivered from 4 recipients and were screened for the transgene by PCR using genomic DNA from the tail. Five newborn piglets were identified as TG founders (F0). Next generation (F1) offspring were produced by natural mating between TG founders and non-TG pigs. Four matings resulted in 37 piglets including 16 TGs. a: including two postnatal deaths. b and c: including one postnatal death.

**Fig. 1.** Identification of transgenic piglets and determination of transgene copy number. A) Schematic diagram of the microinjected transgene. The arrows denote the annealing sites of the two sets of primers used for PCR analysis. The drawing is not proportional to the actual scale. B) Genomic DNAs from the tails of piglets (lanes 1 to 26) were amplified using specially designed PCR primers. In 2, 7, 8, 16 and 17 founders, the transgenes were detected. The expected sizes of the two amplified bands of the transgene are indicated by 1,085 bp and 605 bp. C) Southern blot analysis to determine the copy number of the transgene in transgenic pigs. The arrow at the right indicates a 1.8 kb transgene liberated from the genomic DNA of the transgenic pigs by *Pst I* digestion.
jected to MALDI-TOF analysis. Bands 1 and 2 were matched to the alpha s2-casein-pig and vWF (Homo sapiens) and vWF (Sus scrofa), respectively, and band 3 was matched to vWF prepropeptide (Homo sapiens), vWF precursor (Sus scrofa), vWF (Pteronotus parnellii) and alpha s2-casein-pig (Table 2).

Since the formation of multimeric structures is one of the major characteristics of hvWF in vivo, we analyzed the multimeric structure of rhvWF derived from the TG pig milk by using a 1.5% horizontal SDS-agarose gel (Fig. 5). Although the multimeric structure of rhvWF in the milk of the TG sows was retained until heparin chromatography was performed, formation of the multimeric structure was disturbed after the SP elution step.

Two of our main concerns with regard to this procedure were the yield of the purification product obtained and whether the final product retains its biological activity. It is known that vWF plays a critical role in maintaining hemostasis by controlling the assembly of FVIII [25]. To evaluate the binding of FVIII to human plasma-vWF and TG pig milk-rhvWF, we measured the capture of FVIII by 200 ng/ml of rhvWF bound to plates. The results showed that the binding ability of FVIII to each vWF was similar for the human plasma and TG-pig milk (Fig. 6).

**Discussion**

Here, we report the generation of TG pigs expressing high levels of the rhvWF protein in their milk under the control of the bCSN1S1 promoter. Expression of the rhvWF protein in the milk of TG pigs when driven by the bCSN1S1 promoter appears to be more efficient than that when driven by other promoters such as the mouse WAP, β-lactoglobulin [26] and bovine α-lactalbumin promoters [27]. The rhvWF protein isolated from the milk of these TG pigs exhibited multimeric structures as well as the ability to bind
PRODUCTION OF RHVWF IN THE MILK TG PIGS

Currently, the restricted production of vWF from cultured cells and human plasma limits its use. Our bioreactor system can generate high levels of functional rhvWF and can thus increase the production efficiency and decrease the production cost to a reasonable level. The present study produced the first example of a successful TG pig bioreactor producing rhvWF in milk that may be used for further biomedical purposes.

Table 2. Identification of tryptic peptides from TG-pig milk

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Protein name</th>
<th>Ion score</th>
<th>Accession no.</th>
<th>Mr</th>
<th>pl</th>
<th>Seq. cov. (%)</th>
<th>Best peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alpha s2-casein - pig</td>
<td>58</td>
<td>q1 I 477186</td>
<td>27724</td>
<td>5.94</td>
<td>9</td>
<td>FPQYLQALYQAQIVMNPDQ</td>
</tr>
<tr>
<td>2</td>
<td>Von Willebrand factor (Homo sapiens)</td>
<td>143</td>
<td>q1 I 553810</td>
<td>107898</td>
<td>5.12</td>
<td>5</td>
<td>LLDVFLDLGSSR</td>
</tr>
<tr>
<td>3</td>
<td>Von Willebrand factor (Sus scrofa)</td>
<td>68</td>
<td>q1 I 12230718</td>
<td>284247</td>
<td>5.38</td>
<td>0</td>
<td>YTLFQIFSK</td>
</tr>
<tr>
<td>4</td>
<td>Von Willebrand factor prepropeptide (Homo sapiens)</td>
<td>654</td>
<td>q1 I 340361</td>
<td>237661</td>
<td>5.44</td>
<td>9</td>
<td>LTQVSVLYQGSITTDVPW</td>
</tr>
<tr>
<td>5</td>
<td>Von Willebrand factor precursor (Sus scrofa)</td>
<td>170</td>
<td>q1 I 12230718</td>
<td>284247</td>
<td>5.38</td>
<td>1</td>
<td>LLDVFLDLGSSK</td>
</tr>
<tr>
<td>6</td>
<td>Von Willebrand factor (Pteronotus parnelli)</td>
<td>103</td>
<td>q1 I 32140141</td>
<td>43540</td>
<td>6.20</td>
<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>Alpha s2-casein - pig</td>
<td>63</td>
<td>q1 I 477186</td>
<td>27724</td>
<td>5.94</td>
<td>8</td>
<td>FPQYLQALYQAQIVMNPDQ</td>
</tr>
</tbody>
</table>

* The ion score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Individual ion scores >48 indicate identity or extensive homology (P<0.05). b Accession number is the NCBIinr database number. c Sequence converge.

Fig. 5. Multimeric structure analysis of rhvWF derived from the milk of transgenic swine. The vWF multimeric structure was analyzed by SDS-1.5% agarose gel electrophoresis in each stage of purification. The multimeric structure of rhvWF in the TG sow milk was destroyed after SP elution. F: fat elimination. C: cryoprecipitation. ASP: ammonium sulfate precipitation. D: Factor VIII deficient human plasma. H: heparin

Fig. 6. Comparisons of Factor VIII binding ability between vWFs derived from human plasma and TG milk. The ability of vWF (from human plasma) and rhvWF (from TG milk) to bind FVIII was determined by ELISA using a biotinylated anti-FVIII mAb to detect bound FVIII, as described under Materials and Methods. The open triangles (△) represent human plasma, and the closed circles (●) represent TG milk using 200 ng/ml of vWF coating.
research as well as treatment of patients suffering from blood disorders. Furthermore, our TG line of pigs could be used for various studies, including gene therapy research and clinical trials of rhVWF. The efficiency of producing TG animals is an important factor in transgenic technology. Although numerous investigators have succeeded in generating TG pigs, the efficiency of producing these TG pigs by the microinjection method has only been approximately 1% [28, 29]. Furthermore, many of the founder animals are mosaic and transmit the transgene to their offspring at low frequencies. In our previous study on TG pigs, 19.2% newborn piglets received the transgene as founders (F0), and 43.2% of the F0 animals transmitted the transgene to F1 offspring. In this study, the gene transfer rates increased considerably. Furthermore, the transgene copy number determined by southern blot analysis strongly suggests that transgenes were transmitted stably to their progeny (Fig. 1C).

As shown in Table 2, although band 2 was not detected in skimmed milk diluted 8- and 16-fold, the peptides derived from human vWF are included in bands 2 and 3, which indicates that they are expressed by the transgene. On the other hand, vWF is a multimeric adhesive glycoprotein that serves as a carrier for factor VIII in plasma and mainly functions by binding to other proteins, particularly FVIII. Although we examined the multimeric structure and FVIII-binding ability of rhVWF by using the vWF protein isolated from the milk of TG sows and purified in a simple manner, we did not assess its biological activity in a cell culture system. The multimeric structure of rhVWF was retained until the SP elution step, following which it was disturbed. The physiological characteristics of these TG animals are still under investigation; however, we did not encounter any serious obstacles to their breeding. Moreover, we intend to produce other unique TG swine by mating an over, we intend to produce other unique TG swine by mating an

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