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Sperm mediated human coagulation factor VIII gene transfer and expression in transgenic mice

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Summary

Principles: B-domain deleted human coagulation factor VIII cDNA (BDD-*hFVIII*cDNA) transgenic mice were produced by using sperm mediated gene transfer (SMGT). The transcription and expression of human FVIII in transgenic mice were also investigated.

Methods: Sperm were isolated from caudae epididymides of male C57BL/6 mice and transfected with linearized RC/RSV-BDD-*hFVIII*cDNA plasmid, and subsequently used to fertilize female mice via artificial insemination *in vivo*. After birth, F0 progeny were identified by PCR and Southern blotting for BDD-*hFVII-I*cDNA transgenic mice. F1 progeny were subsequently derived from a male transgenic F0 mouse and a normal C57BL/6 female mouse. The F1 progeny were then identified as BDD-*hFVII-I*cDNA transgenic mice by Southern blotting. The transcription and expression of BDD-*hFVIII*cDNA in transgenic mice were determined by northern blotting, western blotting and immunohistochemical staining. Blood was also collected from both F0 and F1 progeny to detect hFVIII:Ag and anti-hFVIII inhibitors.

Results: A total of 9 F0 and 8 F1 progeny were delivered, in which 3 F0 and 2 F1 progeny were identified to have BDD-*hFVIII*cDNA. The transcription and expression of BDD-*hFVIII*cDNA were found to exist in the liver and kidneys of all transgenic mice. HFVIII:Ag in plasma of the transgenic F0 progeny was 31.95 ng/ml, 23.52 ng/ml and 26.36 ng/ml respectively, whilst the F1 transgenic mice showed results of 18.82 ng/ml and 12.16 ng/ml. Anti-hFVIII inhibitors were negative in both F0 and F1 progeny.

Conclusions: Human FVIII gene transgenic mice can be produced by the SMGT technique and express human FVIII protein in their bodies.

Key words: sperm mediated gene transfer; artificial insemination; transgenic mouse; coagulation factor VIII; gene expression

Introduction

Hemophilia A is an inherited, sex-linked bleeding disease resulting from a defective or deficient coagulation factor VIII (FVIII). With an incidence ratio of 1:5000 in male births, hemophilia A comprises the majority of hemophilia patients (approximately 80%). Hemophilia A patients suffer from spontaneous bleeding into the large joints and soft tissue, and are at risk for intracranial hemorrhage [1]. Currently, treatment for hemophilia A uses the infusion of either plasma-derived or recombinant FVIII protein for bleeding episodes [2]. However, due to the limited FVIII protein source, acquiring large amounts of human FVIII protein from transgenic animals is a new prospective strategy. For a long time, the production of transgenic animals has re-

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lied predominantly on the microinjection technique, in which the gene of interest is introduced into the male pronuclei of the fertilized eggs by using microinjection [3]. Interestingly, sperm may be regarded as more efficient gene transfer carriers due to their particular role in insemination [4]. Therefore, a sperm mediated gene transfer (SMGT) technique could be used to generate transgenic animals capable of producing FVIII protein for purification, in order to produce clotting factor VIII concentrate for human use. In this article, human FVIII gene transgenic mice were produced, using SMGT.

Materials and methods

Animals

A total of 30 C57BL/6 mice (20 females and 10 males) aged 10~12 weeks old (from the Laboratory Animal Center of Medical College of Shantou University, China) were used. All procedures and handling of animals were reviewed and approved by the Animal Care and Use Committee of the Medical College of Shantou University, and conformed to *the Guide for the Care and Use of Laboratory Animals* (No. 2 Document from the Science & Technology Committee, China, 1988). Tissue harvest was performed under general anesthesia.

Preparation of exogenous DNA

*Sca*I linearized RC/RSV-BDD-*bFVIII*cDNA plasmid (from Shanghai Institute of Hematology, China), which contained B-domain deleted human coagulation factor VIII cDNA (BDD-*bFVIII*cDNA), was purified using a QIAquick DNA purification kit (QIAGEN, Gene Company Limited, Hongkong, China) and stored at –20 °C.

Collection of sperm

Sperm were isolated from caudae epididymides of male C57BL/6 mice [5]. Seminal fluid was removed by washing sperm in culture medium [1 liter contained 11.25 g glucose, 10 g sodium citrate ($2\bullet H_2O$), 4.7 g EDTA ($2\bullet H_2O$), 3.25 g citric acid (H_2O), 6.5 g Tris base adjusted to pH 7.4] supplemented with 6 mg/ml bovine serum albumin (BSA) pre-warmed to 37 °C. After this treatment, the medium was kept at room temperature (RT). Semen was incubated for 5 min, transferred to 10-ml tubes and spun at 800 x *g* for 10 min (25 °C). Supernatants were then aspirated prior to sperm being suspended and spun again at 800 x *g* for 10 min (16 °C) and before being discarded. Sperm cells were re-suspended, and an aliquot of the sperm suspension was then used to determine sperm concentration and the percentage of spermatozoa that were motile. The suspension was then adjusted so that there were approximately 1×10^8 spermatozoa/ml [5].

Uptake of exogenous DNA by sperm

*Sca*I linearized RC/RSV-BDD-*bFVIII*cDNA plasmid (5 μg) was dissolved in 80 μl culture medium and mixed with 20 μl SuperFect Transfection Reagent (QIAGEN). Samples were then incubated at ambient temperature for 10 minutes to form complexes. The complexes were then incubated for a further 30 minutes at 37 °C, 5% CO_2 with 100 μl sperm suspension (1×10^8 spermatozoa/ml). Prior to artificial insemination, the sperm suspension was diluted to $1\sim2 \times 10^6$ motile spermatozoa/0.05 ml [6].

Artificial insemination (AI)

A total of 18 female C57BL/6 mice were hormonally primed with an i.p. injection of 5 IU pregnant mare serum gonadotrophin (PMSG) (Sigma, Sigma-Aldrich China Inc., Shanghai, China). Mice were then further exposed to an i.p. injection of 5 IU human chorionic gonadotrophin (hCG) (Sigma) 48 hours later. Female mice were subsequently inseminated 11~11.5 hours after hCG injection, immediately preceding ovulation. A volume of 0.05 ml of sperm suspension was then transferred into the cervix via the vagina with a bent, blunted 18-gauge needle [6].

Tail DNA extraction and identification for BDD-hFVIIIcDNA transgenic F0 progeny

Tail DNA extraction

After cyesis, the female mice delivered progeny, which were referred to as F0 progeny. In the fourth week after the birth of F0 progeny, a segment of tail (approximately 0.5~1.0 cm) from each F0 progeny was taken and homogenized in 2 ml TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Genomic DNA was extracted with phenol/chloroform and ethanol precipitation.

Identification by polymerase chain reaction (PCR)

A pair of oligodeoxynucleotides with the following sequences were used: 5′-GAATTCAAGACACCCTAG CACTAGGC-3′ (upstream), 5′-CCATGAGCAGGGTT CAGTGTG-3′ (downstream). These oligodeoxynucleotides amplified a specific 735-bp fragment spanning the deleted B-domain of BDD-*bFVIII*cDNA. While another pair of oligodeoxynucleotides with the following sequences was also used to amplify a 555-bp fragment from mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as inner control: 5′-TGTCGTG GAGTCTACTGGTGTCTTC-3′ (upstream), 5′-TC CTCAGTGTAGCCCAAGATGC-3′ (downstream). F0 progeny were examined by PCR with one microgram of genomic DNA as a template in a 25 μl reaction volume containing 2.5 mM $MgCl_2$, 250 μM of each dNTP, 100 pM of each primer and 2.5 units of thermostable DNA polymerase. Another tube containing *Sca*I linearized RC/RSV-BDD-*bFVIII*cDNA plasmid as a template in the same PCR buffer solution served as a positive control. The PCR mixture containing all components was denatured at 95 °C for 5 minutes, as performed via 35 cycles of PCR (94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute and 30 seconds), and then incubated at 72 °C for 10 minutes. PCR reactions were conducted in triplicate. PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel.

Identification by Southern blotting

Mice tail DNA was incubated with *Bgl*II restriction endonuclease and fractionated through a 0.8% (w/v) agarose gel. The gel was then blotted onto a nitrocellulose membrane, which was incubated for 2 hours at 80 °C to fix the DNA. Furthermore, the membrane was prehybridized for 4 hours and hybridized overnight with linear BDD-*bFVIII*cDNA probe labeled with α-^{32}P dCTP (TaKaRa Biotechnology Co., Ltd. Dalian, China) in hy-

bridization buffer [6 × SSC (sodium chloride/sodium citrate buffer), 5 × Denhardt's solution, 0.5% (w/v) SDS (sodium dodecylsulphate), 50% (v/v) formamide, 100 μg ml^{-1} salmon sperm DNA]. After hybridization, the membrane was washed successively with 2 × SSC and 0.5% SDS for 5 minutes at room temperature, 2 × SSC and 0.1% SDS for 15 minutes at room temperature, 0.1 × SSC and 0.5% SDS for 30 minutes at 42 °C, and then with 0.1 × SSC and 0.1% SDS for 30 minutes at 65 °C. Finally, the membrane was exposed to a Kodak phosphorimaging screen for three days and visualized using a Molecular Imager FX (Bio-Rad Pacific Ltd, Shanghai, China).

Delivery of F1 progeny and identification for BDD-hFVIIIcDNA transgenic mice by Southern blotting

BDD-*hFVIII*cDNA transgenic F0 male progeny, the founder mouse, was mated with a normal female C57BL/6 mouse and delivered progeny, which were referred to as F1 progeny. After 4 weeks from the birth of the F1 progeny, tail DNA was extracted and identified by Southern blotting for BDD-*hFVIII*cDNA transgenic mice.

Northern blot analysis

In order to investigate the transcription of BDD-*hFVIII*cDNA in transgenic mice, transgenic mice and a non-transgenic mouse (control animal) were sacrificed, and the liver and kidneys harvested. Total RNA was isolated from the liver and kidneys by using Trizol reagent (GIBCO BRL) according to the manufacturer's instructions, and separated on 1% (w/v) agarose gel containing 1 × MOPS buffer [20 mM 3-(N-morpholino) propanesulfonic acid, 8 mM sodium acetate and 1mM EDTA] and 2.2 M formaldehyde. Total RNA was then transferred by capillary action to a nitrocellulose membrane. The membrane was prehybridized and hybridized as described in Southern blotting. Prehybridization and hybridization solutions were 50% (v/v) formamide, 5 × SSC, 2 × Denhardt's solution, 0.1% (w/v) SDS, and 100 μg ml^{-1} salmon sperm DNA respectively. After being washed with 2 × SSC and 0.5% (w/v) SDS at room temperature for 5 minutes, 1 × SSC and 0.1% SDS at 42 °C for 30 minutes, 0.1 × SSC and 0.5% SDS at 56 °C for 30 minutes, the membrane was exposed and analyzed as previously described in Southern blotting.

Immunoprecipitation and Western blotting

Rabbit anti-human FVIII monoclonal antibody (from Shanghai Institute of Hematology, China) included antibody reacting with heavy chain (MAb-hFVIII-H) and with light chain (MAb-hFVIII-L). The epitope specificity of MAb-hFVIII-H was directed against the acidic region 336–372 between A1 and A2 domain of hFVIII, and that of MAb-hFVIII-L was directed against sequence 1811–1818 in A3 domain of hFVIII [7]. Since the epitope sequences reacted in human FVIII were quite different from those in murine FVIII, rabbit anti-human FVIII monoclonal antibody used to detect human FVIII had no cross reactivity to murine FVIII. Rabbit anti-human FVIII monoclonal antibody was bound to a recombinant protein A-Sepharose 4B conjugate (Zymed, Golden Bridge International, Inc., Beijing, China) by mixing 100 μl of 50% bead suspension with 1.5 μl rabbit anti-human FVIII antibody and 400 μl PBS-T [10 mM NaH_2PO_4 (pH 7.2), 0.15 mM NaCl, 0.05% Tween-20]. After 2 hours incubation, the beads were washed and re-suspended to 500 μl with PBS-T. The liver and kidneys from transgenic mice and non-transgenic mice (control animals) were homogenized in 200 μl RIPA buffer [20 mM Tris-HCl (pH 7.4), 1% Triton, 100 μM PMSF (phenylmethylsulfonyl fluoride), 0.1% SDS] respectively. After centrifugation, the supernatant was mixed with 100 μl of the protein A-Sepharose 4B-anti-human FVIII antibody suspension and incubated overnight at 4 °C. The beads were then washed three times with PBS-T and the bound protein was eluted with sample buffer for SDS/PAGE (sodium dodecylsulphate/polyacrylamide gel electrophoresis). After being separated by electrophoresis on 6% SDS-PAGE, protein was transferred to a nitrocellulose membrane by using a semidry transfer apparatus (Bio-Rad). Subsequently, the membrane was blocked with 2% BSA in TBS-T [10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Tween-20], and then incubated at 25 °C for 1 hour with the primary rabbit anti-human FVIII antibody (diluted 1:1000). After incubation, the membrane was successively washed with TBS-T, incubated for 1 hour with alkaline phosphatase (AP)-conjugated secondary antibody [goat anti-rabbit IgG (Santa Cruz, Gene Company Ltd., Shanghai, China)] and washed five times with TBS-T. Finally, 5-bromo-4-chloro-3-indolephosphoric acid (BCIP, 0.25 mg/ml) and nitroblue tetrazolium (NBT, 0.25 mg/ml) (Sigma, Sigma-Aldrich China Inc., Shanghai, China) were added as substrates. Recombinant full-length human FVIII protein containing B-domain (rhFVIII, Baxter Biosciences Corporation, Shanghai, China) purified in parallel was used as a standard.

Immunohistochemical staining of human FVIII in mice tissue

The livers, kidneys and lungs of transgenic progeny were fixed with neutral buffered 10% formalin and embedded in paraffin. Paraffin sections (5-μm-thick) were pre-incubated with 0.1% hydrogen peroxide for 10 minutes at room temperature. Sections were then stained with rabbit anti-human FVIII monoclonal antibody (identical as to that in western blotting, diluted 1:1000) for 1 hour at room temperature, followed successively by additional staining with biotinylated goat anti-rabbit IgG and the avidin-biotin complex (ABC kit; Vector Laboratories, Jingmei Biotech Co., Ltd. Shenzhen, China). After adding 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen, sections were flooded with TE buffer (10 mM Tris-Cl, 1mM EDTA, pH 7.5), and the nuclei were counterstained with hematoxylin. Finally, sections were dehydrated and mounted. Negative controls were prepared in parallel by replacing the primary antibody with normal rabbit IgG.

Antigen assay of human FVIII in plasma of transgenic mice (competitive ELISA)

Blood was collected from both F0 and F1 progeny and plasma was isolated and analyzed for human FVIII antigen by competitive enzyme-linked immunosorbent assay (ELISA) [8]. ELISA 96-well plates (Nalge Nunc Inc., Shanghai Kou Hing Hong Scientific, Shanghai, China) were coated with recombinant factor VIII (rFVIII) (50 ng/well) in 50 μl of 50 mM Na_2CO_3, pH 9.6, left overnight at 4 °C. After three washings with PBS-T, the plates were incubated for 1 hour with 2% BSA in TB [20 mM Tris (pH 7.6), 0.15 mM NaCl] at ambient temperature to block any nonspecific binding. Samples were then washed three times with PBS-T. In a separate vessel, rabbit anti-human FVIII antibody (identical to that in Western blotting and immunohistochemical staining) was incubated with the competing antigen (the diluted sample of 0 to 50 ng rFVIII) overnight in 50 μl TB plus 0.1% BSA at 4 °C. A volume of 50 μl from each vessel was then added to each well for 3 hours incubation at ambient temperature, followed by washing plates a total of seven times with PBS-T. The detecting antibody (50 μl of goat anti-rabbit IgG conjugated to AP diluted 1:1000

with 0.1% BSA in TB) was then added to each well and incubated for 60 minutes at 37 °C. After five washings with PBS-T, 100 µl BCIP/NBT (SouthernBiotech, Neo-Bioscience Technology Company, Shenzhen, China) was then added to each well as substrate. The enzymatic reaction was stopped by adding 0.5 M H_2SO_4. The amount of the expressed BDD-hFVIII could be determined by reading the plate on a microtest plate reader at a wavelength of 450 nm. The rFVIII standard curve was constructed by plotting the mean absorbance value calculated for each sample with different concentrations.

Detection of anti-hFVIII inhibitors in plasma of transgenic mice

The Bethesda inhibitor assay (BIA) [9] was used to detect anti-hFVIII inhibitors in both F0 and F1 progeny plasma. Briefly, the mice plasma was incubated at 55 °C for 30 minutes to inactivate endogenous murine FVIII. Serial dilution of the treated mice plasma was then mixed with an equal volume of pooled normal human plasma (UCRP, Shanghai Fisher Scientific, Shanghai, China) and incubated at 37 °C for 2 hours. Activated partial thromboplastin time (APTT) was performed to determine the residual FVIII activity in the UCRP incubated with the inactivated mice plasma. The anti-hFVIII inhibitor titer was calculated from the residual FVIII activity of each sample according to the established BIA standard curve.

Results

Delivery of F0 and F1 progeny

A total of 5 out of the 18 female mice which received AI, became pregnant and delivered 9 young (F0 progeny). The F0 progeny contained 4 females and 5 males and were given the serial numbers 1 to 9. Whilst 8 F1 progeny were delivered (5 females and 3 males) and given serial numbers 6-1 to 6-8.

Identification for BDD-*hFVIII*cDNA transgenic mice by PCR and Southern blotting

PCR

F0 mice, in which the 735-bp specific fragment of BDD-*hFVIII*cDNA was amplified by PCR from tail DNA sample, were regarded as BDD-*hFVIII*cDNA transgenic ones. From 9 F0

Figure 1

Identification for BDD-hFVIIIcDNA transgenic mice by PCR and Southern blotting.
A PCR of the tail DNA from F0 progeny. A 735-bp specific fragment of BDD-*hFVIII*cDNA was amplified from the tail DNA of samples3, 6 and 8 F0 progeny respectively (lane 3, 6 and 8), and no fragment of BDD-*hFVIII*cDNA was amplified from the tail DNA of samples1, 2, 4, 5, 7 and 9 F0 progeny (lane 1, 2, 4, 5, 7 and 9). Lane M was DL2000 DNA Marker. Lane 10 showed a 735-bp fragment was amplified from *Sca*I linearized RC/RSV-BDD-*hFVIII*cDNA plasmid, which served as positive control. Lane 11 were PCR products from the tail DNA of a normal C57BL/6 mouse, no fragment of BDD-*hFVIII*cDNA was amplified either. A 555-bp fragment of mouse GAPDH gene (inner control) was amplified from the tail DNA of all mice.
B Map of the RC/RSV-BDD-*hFVIII*cDNA construct. RC/RSV-BDD-*hFVIII*cDNA was 9838-bp long, including the BDD-*hFVIII*cDNA (4666-bp) and the *LTR-RSV* region (422-bp). Cloning sites were shown on the schema. The construct was linearized with *Sca*I. *Bgl*II digestion of RC/RSV-BDD-*hFVIII*cDNA yielded three bands, 455-bp, 2320-bp and 7063-bp.
C Southern blotting of the tail DNA from samples 1 to 9 F0 progeny (lane 1–9), DNA from control mouse plus *Sca*I linearized RC/RSV-BDD-*hFVIII*cDNA (lane 10) and DNA from a normal control C57BL/6 mouse (lane 11). The tail DNA was digested with *Bgl*II, run on 0.8% (w/v) agarose gel, and blotted and probed with the BDD-*hFVIII*cDNA minigene. The two bands of 2320-bp and 7063-bp generated by *Bgl*II were in lane 10. After *Bgl*II digestion of the transgenic mouse genomic DNA, the following pattern could be predicted to occur: (*i*) 1 integration site, 1 copy: an internal 2320-bp band and one band of >7063-bp; (*ii*) 1 integration site, 2 copies, head-to-tail orientation: two copies of the 2320-bp band plus one 7518-bp band and one band of >7063-bp; (*iii*) 1 integration site, 2 copies, head-to-head orientation: two copies of the 2320-bp band and two bands of >7063-bp; (*iv*) 1 integration site, 2 copies, tail-to-tail orientation: two copies of the 2320-bp band and one 14126-bp band; (*v*) more than one integration site, 1 copy in each: multiple copies of the 2320-bp band and multiple bands of >7063-bp of different sizes. Combinations of the above cases were also possible. A further degree of complexity could result from rearrangements of DNA after integration. Lane 3 showed bands of >20, 9.0, 7.5 and 2.3 kb, pattern was compatible with array (*ii*) or with a combination of patterns (*i*) and (*ii*) or (*ii*) and (*v*). Lane 6 showed bands of >20, 9.0 and 2.3 kb, pattern was compatible with array (*iii*) or with a combination of patterns (*i*) and (*iii*) or (*iii*) and (*v*). Lane 8 showed bands of >20, 14, 4.1 and 2.3 kb, pattern was compatible with array (*iv*) or with a combination of (*i*) and (*iv*) or (*iv*) and (*v*). No bands were visible in lane 1, 2, 4, 5, 7 and 9.
D Southern blotting of DNA from the tail of samples 6-1 to 6-8 F1 progeny (lane 1-8), DNA from control mouse plus *Sca*I linearized RC/RSV-BDD-*hFVIII*cDNA (lane 9) and DNA from a normal control C57BL/6 mouse (lane 10). The two bands of 2320-bp and 7063-bp generated by *Bgl*II were in lane 9. Both lane 2 and lane 5 showed bands of >20, 9.0 and 2.3 kb, which were the same integration pattern as the founder, sample 6 F0 progeny. No bands were visible in lane 1, 3, 4, 6, 7, 8 and 10.

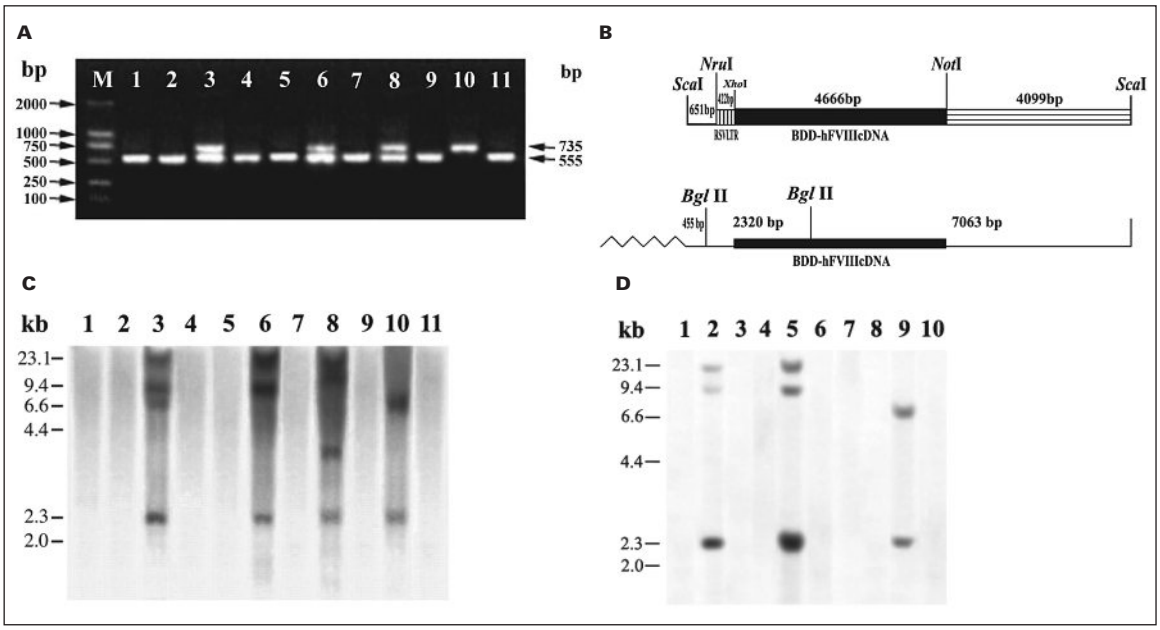


Figure 2

Northern blotting of BDD-hFVIIImRNA in tissues of transgenic mice.
A Northern blotting from transgenic samples 3 F0 progeny (lane 1 and 2), 6 F0 progeny (lane 3 and 4), 8 F0 progeny (lane 5 and 6) and 1 non-transgenic F0 progeny (lane 7 and 8). Lane 1, 3, 5 and 7 were from liver samples; lane 2, 4, 6 and 8 were from the kidneys. Ten micrograms of RNA per lane were run onto a 1% formaldehyde agarose gel, blotted, and hybridized with the BDD-*hFVIII*cDNA probe. BDD-*hFVIII*mRNA existed in the livers and kidneys of 3 transgenic F0 progeny (lane 1-6).
B Northern blotting from transgenic samples 6-2 F1 progeny (lane 1 and 2), 6-5 F1 progeny (lane 3 and 4) and 6-6 non-transgenic F1 progeny (lane 5 and 6). Lane 1, 3 and 5 were from liver samples; lane 2, 4 and 6 were from the kidneys. BDD-*hFVIII*mRNA existed in the livers and kidneys of 2 transgenic F1 progeny.

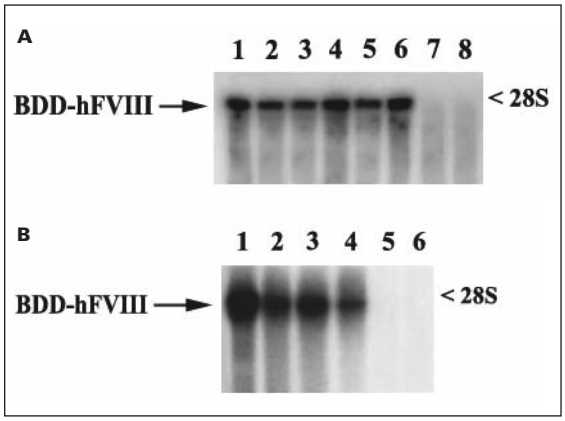
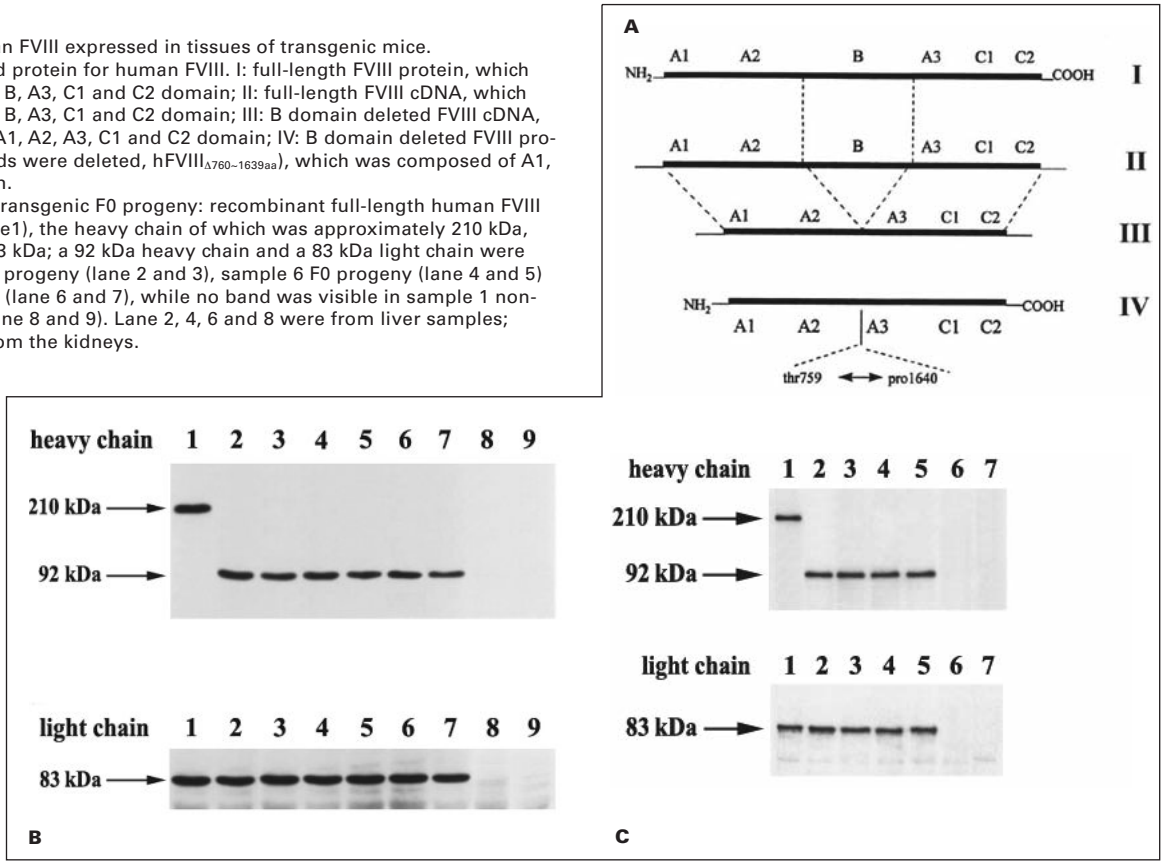


Figure 3

Western blotting of human FVIII expressed in tissues of transgenic mice.
A Two forms of cDNA and protein for human FVIII. I: full-length FVIII protein, which was composed of A1, A2, B, A3, C1 and C2 domain; II: full-length FVIII cDNA, which was composed of A1, A2, B, A3, C1 and C2 domain; III: B domain deleted FVIII cDNA, which was composed of A1, A2, A3, C1 and C2 domain; IV: B domain deleted FVIII protein (760~1639 amino acids were deleted, $hFVIII_{\Delta 760\sim1639aa}$), which was composed of A1, A2, A3, C1 and C2 domain.
B Western blotting from transgenic F0 progeny: recombinant full-length human FVIII containing B-domain (lane1), the heavy chain of which was approximately 210 kDa, and the light chain was 83 kDa; a 92 kDa heavy chain and a 83 kDa light chain were presented in sample 3 F0 progeny (lane 2 and 3), sample 6 F0 progeny (lane 4 and 5) and sample 8 F0 progeny (lane 6 and 7), while no band was visible in sample 1 non-transgenic F0 progeny (lane 8 and 9). Lane 2, 4, 6 and 8 were from liver samples; lane 3, 5, 7 and 9 were from the kidneys.
C Western blotting from transgenic F1 progeny: recombinant full-length human FVIII containing B-domain (lane1) presented a 210 kDa heavy chain and a 83 kDa light chain, sample 6-2 F1 progeny (lane 2 and 3) and sample 6-5 F1 progeny (lane 4 and 5) presented a 92 kDa heavy chain and a 83 kDa light chain, while there was no band in sample 6-6 non-transgenic F1 progeny (lane 6 and 7). Lane 2, 4 and 6 were from liver samples; lane 3, 5 and 7 were from the kidneys.



progeny, 3 transgenic mice were identified to have the transgene, with serial numbers 3 (female), 6 (male) and 8 (female) respectively, the overall rate of transgenic production was 33.3% (3/9) (fig. 1A).

Southern blotting

Sperm cells were used as vectors for transferring a 9.8-kb construct, containing BDD-*hFVII-I*cDNA, into F0 progeny by artificial insemination. *Bgl*II cut twice in *Sca*I linearized RC/RSV-BDD-*hFVIII*cDNA plasmid, giving rise to three bands of 455-bp, 2320-bp and 7063-bp. However, since the probe used was the linear BDD-*hFVII-I*cDNA, after hybridization, only 2320-bp and 7063-bp were displayed (fig. 1B).

Fig. 1C showed Southern blots of *Bgl*II digested tail DNA from F0 progeny, as a positive control, DNA from a non-transgenic mouse was mixed with *Sca*I linearized RC/RSV-BDD-*hFVIII*cDNA plasmid. Under our high stringency experimental conditions, different patterns of hybridization bands were displayed in F0 progeny DNA (samples 3, 6 and 8); the 2.3-kb band corresponded to the internal band of the RC/RSV-BDD-*hFVIII*cDNA construct. The other bands represented fragments generated by *Bgl*II digestion of transgenic mouse DNA; their number and sizes were consistent with the bands predicted, considering the patterns (head to head, head to tail and tail to tail) generated by transgene integration in single or multiple sites. It was indicated that 3, 6 and 8 F0 progeny were transgenic animals, in accordance with the results of PCR (fig. 1C).

PCR and Southern blotting identified that the male 6 F0 progeny was BDD-*hFVIII*cDNA transgenic founder. The founder was mated with a normal female C57BL/6 mouse and delivered 8 F1 progeny coded 6-1 to 6-8. Southern blot analysis showed that DNA samples 6-2 to 6-5 F1 progeny displayed hybridization bands, which in-

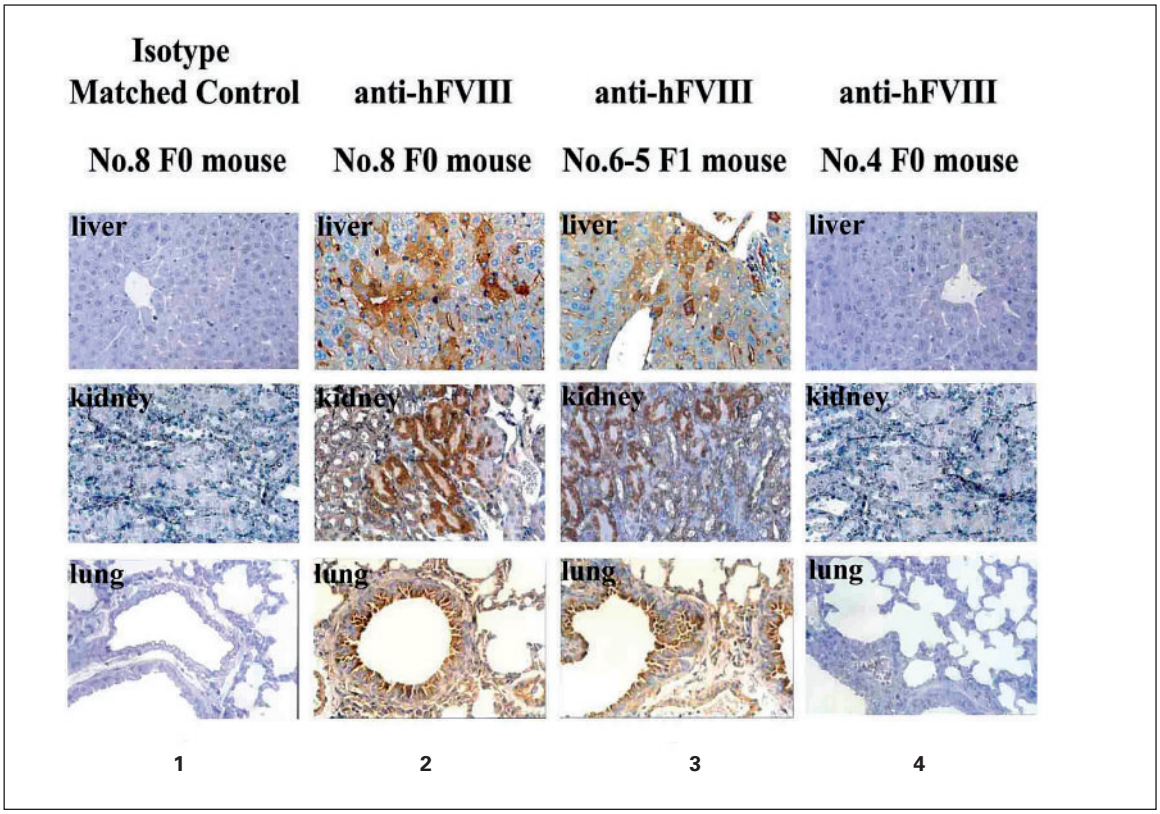


Figure 4

Immunohistochemical staining for human FVIII in tissues from transgenic mice.
Column 2, 3 and 4 were stained with anti-human FVIII antibody, and column 1 was isotype matched control, which was stained in parallel by replacing anti-human FVIII antibody with normal rabbit IgG. Staining was with DAB as chromogen and haematoxyllin restaining, × 400, brown-yellow sites demonstrated positive hFVIII expression.

dicated the same integration patterns presented in the founder. This indicated that 6-2 and 6-5 F1 progeny (both female) were BDD-*hFVIII*cDNA transgenic mice (fig. 1D). No bands were visible in DNA samples from other F1 progeny. Therefore samples 6-2 and 6-5 were BDD-*hFVII-I*cDNA transgenic F1 progeny.

Northern blotting

Northern blot analysis showed that the BDD-*hFVIII*cDNA transgene was transcribed in the livers and kidneys of 3 transgenic F0 and 2 transgenic F1 progeny, while northern blots of non-transgenic progeny tissues prepared in an identical manner were negative. The size of the BDD-*hFVIIIcDNA* transcript was evaluated with respect to approximately 28S rRNA (fig. 2).

Western blotting

Western blotting showed that B-domain deleted human FVIII (760~1639 amino acids deleted, $hFVIII_{\Delta 760\sim 1639aa}$) were expressed in the livers and kidneys of 3 transgenic F0 and 2 transgenic F1 progeny. $HFVIII_{\Delta 760\sim 1639aa}$ also existed in the mode of heterodimers consisting of a heavy chain and a light chain [10]. We used recombinant full-length human FVIII (containing B domain) herein as control. The full-length human FVIII (containing B domain) protein consisted of 2332 amino acids that exhibited 6 distinct structural domains, A1, A2, A3, B, C1 and C2. All the domains were arranged in the order A1-A2-B-A3-C1-C2 (fig. 3A), in which A1-A2-B made up the heavy chain with molecular weight of approximately 210 kDa, and A3-C1-C2 formed the light chain with molecular weight around 83 kDa [10]. In our study, B domain (760~1639 amino acids) was deleted, therefore, the heavy chain of human $FVIII_{\Delta 760\sim 1639aa}$ was composed of A1-A2 domains, when detected with MAb-hFVIII-H, the molecular weight of the heavy chain of $hFVIII_{\Delta 760\sim 1639aa}$ was approximately 92kDa, when detected with MAb-hFVIII-L, the light chain of human $FVIII_{\Delta 760\sim 1639aa}$ was approximately 83kDa. There was, however, no human FVIII expressed in non-transgenic progeny tissues prepared in an identical manner (fig. 3 B and 3C).

Immunohistochemical staining

In immunohistochemical staining, control sections incubated with normal rabbit IgG instead of the primary antibody showed no nonspecific binding. Staining with the rabbit anti-human FVIII monoclonal antibody, did suggest that there was expression of human FVIII in the livers, kidneys and lungs of all BDD-*hFVIII*cDNA transgenic progeny. The sections showed that human FVIII expressed mainly in cytoplasma. In the liver, it expressed intensively in sinusoidal endothelial cells besides parenchymal cells. While in the kidneys, human FVIII focused in epithelia of tubular, especially in those of distal tubules adjacent to collecting ducts. In the lungs, although it presented in interstitial cells, human FVIII emerged primarily in columnar epithelial cells and goblet cells of terminal bronchiole. No expression of human FVIII was detectable in the same tissue of non-transgenic progeny [fig. 4 showed the immunohistochemical staining in samples 8 (F0 progeny), 6-5 (F1 BDD-*hFVII-I*cDNA transgenic progeny) and 4 (F0 non-transgenic progeny)].

Human FVIII antigen (hFVIII:Ag) and anti-hFVIII inhibitors in plasma of transgenic mice

Human FVIII:Ag in plasma of samples 3, 6 and 8 BDD-*hFVIII*cDNA transgenic F0 mice was 31.95 ng/ml, 23.52 ng/ml and 26.36 ng/ml respectively. There was no detectable human FVIII:Ag in the plasma of any other F0 mice. Human FVIII:Ag in plasma of samples 6-2 and 6-5 BDD-*hFVIII*cDNA transgenic F1 mice were 18.82 ng/ml and 12.16 ng/ml. There was no human FVIII:Ag in the plasma of the other 6 F1 mice. Anti-hFVIII inhibitors were negative in plasma of both F0 and F1 progeny, nor was there any human FVIII:Ag and anti-hFVIII inhibitors in normal mice examined by using the same method.

Phenotype of transgenic mice

The growth of transgenic mice was normal, and no abnormality was found with the transgenic mice such as thrombosis through overexpression of additional FVIII, or premature ageing. In addition, the fertility of the transgenic mice was also normal for that sample 6 BDD-hFVIIIcDNA F0 founder mouse delivered 8 F1 progeny after being mated with a normal female mouse.

Discussion

To date, the production of transgenic animals has relied almost exclusively on the microinjection technique. However, this technique is relatively complex, extremely time consuming, costly and characterized by low efficiency [11, 12]. In addition, it also results in random integration of exogenous DNA into host chromosome, which causes insertional mutations and variable expression patterns in the transgenic progeny due to position effects [11]. Several years ago, SMGT was described as an alternative technique for the production of transgenic animals [13], in which the gene of interest is taken up by sperm cells in vitro and subsequently integrated into the sperm DNA. Successful transgenesis occurs after fertilization by admixture of the DNA-loaded sperm cells with the eggs *in vitro* or simply by *in vivo* artificial insemination [14, 15]. These studies have been confirmed in a number of laboratories that have reported successful SMGT in several species such as equine, zebrafish, mice, pigs, golden hamsters, silkworms, chickens and rabbits [16–22]. Hence, previous reports and our study demonstrate that SMGT is feasible in creating transgenic animals. In our study, 3 of 9 mice generated by using SMGT were transgenic, the efficient rate was 33.3% (3/9), which was consistent with the efficiency of SMGT reported in previous publications (37.5% in pigs, 33% in mice and 57.1% in rabbits respectively) [18, 22]. However, the efficiency in transgenic mice generated by using microinjection was only 0.5~4% [11], which was lower than that by using SMGT.

Unlike in the microinjection technique, the binding, internalization and possible integration of exogenous DNA into the sperm in SMGT technique are not random events. Sperm cells have a spontaneous tendency to take up exogenous nucleic acid when incubated with foreign DNA [23], and DNA molecules of large size are preferentially taken up as compared to smaller ones [24]. Horan's experimental work indicates that one spermatozoon can take up 3.8×10^2 exogenous DNA molecules [25]. Further polycationic liposomes, such as SuperFect Transfection Reagent used in our study, which presents dendritic structure with a large surface area [26], can condense exogenous DNA molecules into small particles known as polyplexes [27] and favour its uptake. After being taken up by sperm, exogenous DNA is further internalized into the subcellular nuclear scaffold of the sperm head [28], specifically located on the equatorial segment of the head and the periphery of the post-acrosome of sperm, and exists mainly in three parts; the post-head zone, pre-head zone and the whole head of sperm [29]. This interaction is mediated by specific DNA-binding proteins. On the basis of Southern and Western blot analysis, a protein of 30–35 kDa, which is located abundantly on the membrane of sperm head, acts as the potential substrate for exogenous DNA binding and plays a crucial role in the sperm nuclear internalization of sperm-bound DNA [30]. In contrast, seminal plasma contains factors which abolish sperm permeability and exert an inhibitory effect on foreign DNA uptake. The 30–35 kDa DNA-binding protein appears to be the specific target through which the inhibition is mediated. In the presence of the inhibitory factor, the 30–35 kDa protein loses the ability to bind exogenous DNA. Thus, the interaction between exogenous DNA and sperm cells does not appear to be a casual event but, on the contrary, relies on a molecular mechanism based on the cooperation of specific protein factors [30].

In this study, some progeny was non-transgenic for BDD-hFVIIIcDNA. This may be ascribed to problems that arise during the preparation of the sperm, in which damage to the sperm membrane may occur, or residual traces of the inhibitory proteins from seminal fluid counteract the sperm-DNA interaction. Therefore, the conditions described in materials and methods section must be followed with great care. Damage of the sperm membrane may endow the sperm with

changes in capacitation and other functions [31]. In addition, 8 F1 progeny are delivered by natural copulation between sample 6 F0 founder mouse and a normal female, in which only 2 mice carried BDD-hFVIIIcDNA, while another 6 mice are of no transgenics. This indicates that the founder is probably mosaic for BDD-hFVIIIcDNA transgene.

In transgenic F1 progeny, hFVIII:Ag is 18.82 ng/ml and 12.16 ng/ml respectively, which manifests a reduced level comparing with that in transgenic F0 progeny. The probable reason is that apart from BDD-hFVIIIcDNA reduction during transmission, partial transcriptional inactivation of BDD-hFVIIIcDNA may exist. Since human FVIII coagulant activity (hFVIII:C) can not be distinguished from that of mice, it is not possible to detect hFVIII:C in mice. In this study, human BDD-FVIIIcDNA is brought into the uterus by sperm, which combines with ovum to finish insemination and embryogenesis. Since human FVIII antigen is encoded in the sperm-genome and is present at the very beginning of murine immune reconstitution, it might be that a form of immune tolerance protects mice from antibodies against the human antigen, therefore, there are no anti-hFVIII inhibitors detected in plasma of postnatal transgenic mice. Moreover, a normal female mouse delivers usually 8 to 15 progeny for one cyesis, sample 6 BDD-hFVIIIcDNA F0 founder mouse was mated with a normal female mouse and delivered 8 F1 progeny, this implies that the fertility of transgenic mice generated by SMGT is not seriously affected.

In this study, only two generations of transgenic mice without phenotype abnormality were produced by SMGT with the exogenous BDD-hFVIIIcDNA transmitted to the second progeny, however, some problems still require intense research. For example, what was the fate of the sperm's endogenous DNA during fertilization after the exogenous DNA was bound and internalized by sperm cells? How was the possibility of mutation and/or rearrangement of the sperm host chromosome after the exogenous DNA integration? Could the transgene maintain its integrity through several generations to produce a functional FVIII protein? This should be pursued in future research.

From the current findings, it is concluded that B-domain deleted human FVIII cDNA transgenic mice can be produced by SMGT technique and express human FVIII protein in their bodies. Although some limitations and doubts still remain with SMGT in transgenic animal research, decades of hard work suggest that SMGT could become very attractive in the future because of its simplicity and lower cost. Hopefully, this study will provide useful information for the research of human FVIII transgenic livestock (such as sheep and cattle), which serve as bioreactor to produce human FVIII protein.

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