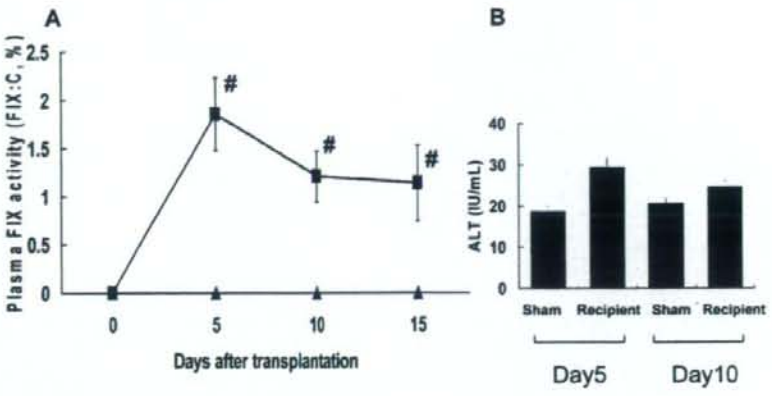

FIGURE 1. Plasma FIX activity (FIX:C) and alanine aminotransferase (ALT) levels of FIX-KO mice after hepatocyte transplantation. (A) FIX:C levels in plasma obtained from FIX-KO mice after hepatocyte transplantation (1.5×10^6 cells/mouse) into the liver (■; n=28, 18, and 13 at day 5, 10, and 15, respectively) or sham operation (▲; n=7 at all time points). Pooled normal mouse plasma was used as a standard. #*P* less than 0.05 between groups. (B) Plasma ALT levels of FIX-KO mice following hepatocyte transplantation (n=25 and 18) or sham operation (n=7) at day 5 and 10 of the experiment.

fied by 1-stage clotting assay based on the activated partial thromboplastin time using human FIX-deficient plasma. Normal mouse plasma was used as FIX:C standard. Each measurement was reported after subtraction of the preoperational baseline FIX:C levels. As a result, FIX:C of recipient mice increased to more than 1% and were stably maintained throughout the experimental period (Fig. 1A). The FIX:C levels were significantly higher in the recipient mice when compared with the levels in the sham-operated mice at every time point examined. At day 5, recipient mice showed a small, but insignificant increase in plasma alanine aminotransferase after the transplantation (n=25) compared with the sham-operated mice (n=7). The slight increase in the alanine aminotransferase levels were found to be declined back toward baseline levels at day 10 (Fig. 1B). These results indicated that hepatocyte transplantation into hemophilia B mice could provide a therapeutic effect by producing FIX from the engrafted donor hepatocytes without significant liver injuries.

Histologic detection of transplanted and engrafted hepatocytes was performed by fluorescence in situ hybridization analysis using mouse Y-chromosome specific probe on sections of female FIX-KO recipient liver that received male hepatocytes. The presence of hepatocytes with Y-chromosome signals were confirmed, indicating the transplanted hepatocytes engrafted into the liver parenchyma (figure not shown). It is also important to note that any cell fusion events were not observed.

To enhance the therapeutic production of FIX in the recipient mice, a repeat transplantation of isolated hepatocytes was performed 15 days after the initial procedure in some recipients by infusing 1.2×10^6 hepatocytes into the upper pole of the spleen (n=4). The other remaining recipients (n=5) were examined with only a single transplantation procedure. As shown in Figure 2, the FIX:C values of the FIX-KO mice at day 25 (10 days after the second transplantation) were 0.94%±0.05% and 1.85%±0.09% in the single- and double-transplanted recipient mice, respectively (P=0.038). Similar increases in FIX:C were also observed at day 35 (20 days after the second transplantation) in the double-transplanted group. These data clearly demonstrated that increasing therapeutic effects could be obtained with a repeated transplantation.

We also examined whether the engrafted hepatocytes were capable of transcribing FIX mRNA in the recipient mouse livers. Because shunting of the hepatocytes into the

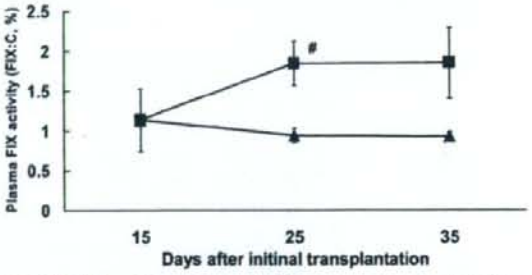

FIGURE 2. Effect of repeated hepatocyte transplantation on plasma FIX:C levels in hemophilia B mice. At day 15, some recipient FIX-KO mice received the second transplantation procedure using 1.2×10^6 hepatocytes (■; n=4), whereas remaining five recipient mice did not receive the second procedure (initial transplantation only, ▲; n=5). #*P* less than 0.05 between groups.

lung had been described in the previous experimental studies (*19*), we also investigated FIX mRNA levels in the lung. Total RNA was extracted from liver, lung, and spleen. Total RNA (1 μg) was reverse transcribed, and the first-strand complementary DNA samples were subjected to quantitative real-time polymerase chain reaction amplification for mouse FIX gene and β-actin gene. Serial dilutions of complementary DNAs of normal mouse liver were used to generate the standard amplification curves. As shown in Figure 3(A), an abundant level of FIX mRNA was detected in the liver, with even higher mRNA expression detected in the livers manipulated with the repeated transplantation. No FIX mRNA signal was detected in the lungs in any of the mouse groups. Incremental expression of FIX was detected in the spleen of single and double hepatocyte transplanted mice, but the levels were markedly lower compared with the livers. We examined the relationship between the FIX:C levels and the liver FIX mRNA levels, and found a direct positive correlation between the two parameters (R^2=0.7214) (Fig. 3B).

Furthermore, we assessed the development of neutralizing antibodies against FIX (FIX inhibitor) by Bethesda method using plasma obtained at killing (*20*). Detectable levels (>0.5 Bethesda U/mL) of FIX inhibitor was not measured

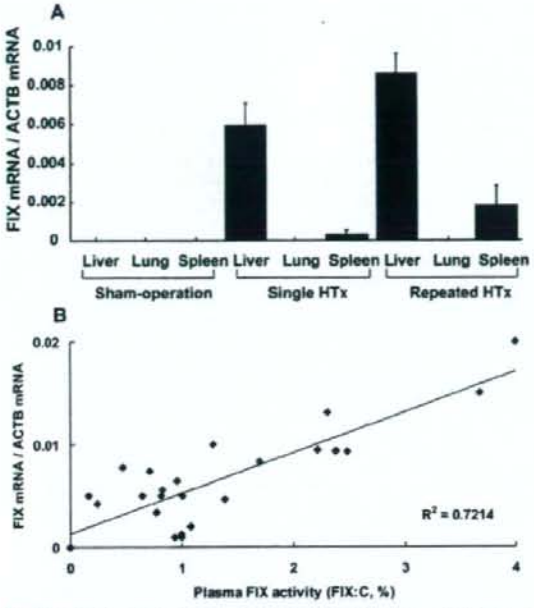

FIGURE 3. Functional engraftment of hepatocytes determined by FIX mRNA expression in the recipient mice. (A) Expression levels of FIX mRNA were determined by quantitative real-time reverse-transcriptase polymerase chain reaction in the liver, spleen, and lung from three experimentally manipulated groups: (1) single hepatocyte transplantation (n=21); (2) repeated hepatocyte transplantation (n=4); and (3) sham-operation (control) (n=7). Each of the FIX mRNA expression values were normalized to a housekeeping gene, β-actin. (B) Relationship between plasma FIX:C levels and FIX mRNA expression levels in the liver of recipient mice. The FIX:C levels of plasma obtained on the day of animal sacrifice were found to correlate with the relative FIX mRNA levels determined in (A) (R^2=0.7214).

in any of recipient mice. This demonstrates that bioengineered FIX produced from the transplanted hepatocytes does not associate with the development of FIX inhibitors.

To investigate the long-term engraftment of hepatocytes, we performed an another set of single transplantation experiment for 12 weeks (n=6), and confirmed long-term persistency of the increased FIX activities at 0.92%±0.22%, 0.78%±0.22%, 0.78%±0.22%, and 0.83%±0.17% at week 2, 4, 8, and 12, respectively.

The present study confirmed the proof-concept feasibility of hepatocyte transplantation as an alternative therapy to treat hemophilia B. The functional engraftment of transplanted hepatocytes within the recipient livers was confirmed by fluorescence in situ hybridization analyses, FIX mRNA expression, and the secretion of functional FIX into the blood circulation. To acquire the proper hemostatic activity, synthesized coagulation FIX requires several posttranscriptional modification steps within the hepatocytes, including cleavage and removal the prepro leader sequence of 46 amino-acids, and γ-carboxylation of the first 12 glutamic acid residues (*21*). For this reason, primary hepatocytes would be more appropriate for transplantation to produce coagulation factors in hemophilia B than other possible types of genetically modified cells expressing FIX.

Previous studies have shown that engrafted hepatocytes within the livers are able to proliferate in response to the regeneration signals occurred by surgical hepatectomy or chronic liver injuries (*22, 23*). Using primary hepatocytes, our group has developed several innovative approaches to create a functional liver system under the kidney capsule or in subcutaneous locations (*13, 15, 16, 24, 25*), and we have clearly demonstrated that these ectopically engrafted hepatocytes also possess the ability for proliferation (*13, 16, 26*). This would be a significant benefit in the use of these hepatocytes, because most of the adult hemophilia B patients presented with chronic hepatitis B and/or C viral infection as a result of treatments with blood-borne contaminated plasma-derived FIX concentrates. Although portion of the transplanted hepatocytes would be infected with hepatitis viruses in the mean time, it would be reasonable to speculate that engrafted hepatocytes will proliferate and expand, which would further increase the therapeutic effects.

In conclusion, the present studies described the feasibility and safety of hepatocyte transplantation as a treatment modality for hemophilia B. Current therapies to treat hemophilia have been confounded with problems, and the present findings represent an important step toward establishing an alternative therapeutic approach for the treatment of not only hemophilia, but other similar genetic disorders affecting the liver.

ACKNOWLEDGMENTS

The authors thank Yuichi Komai, Yuka Bessho, and Sanae Taminishi (Department of Pediatrics, Nara Medical University) for their technical assistance, and Dr. Frank Park (Medical College of Wisconsin) for his critical reading of the manuscript.

REFERENCES

1. Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. *Lancet* 2003; 361: 1801.
2. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med* 2007; 357: 535.
3. Nathwani AC, Davidoff AM, Tuddenham EG. Prospects for gene therapy of haemophilia. *Haemophilia* 2004; 10: 309.
4. Manno CS, Chew AJ, Hutchison S, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 2003; 101: 2963.
5. Manno CS, Pierce GF, Arruda VR, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 2006; 12: 342.
6. Gordon FH, Mistry PK, Sabin CA, et al. Outcome of orthotopic liver transplantation in patients with haemophilia. *Gut* 1998; 42: 744.
7. Ko S, Tanaka I, Kanehiro H, et al. Preclinical experiment of auxiliary partial orthotopic liver transplantation as a curative treatment for hemophilia. *Liver Transpl* 2005; 11: 579.
8. Merion RM, Delius RE, Campbell DA, Jr, et al. Orthotopic liver transplantation totally corrects factor IX deficiency in hemophilia B. *Surgery* 1988; 104: 929.
9. Boost KA, Auth MK, Woitaschek D, et al. Long-term production of major coagulation factors and inhibitors by primary human hepatocytes in vitro: perspectives for clinical application. *Liver Int* 2007; 27: 832.
10. Fisher RA, Strom SC. Human hepatocyte transplantation: Worldwide results. *Transplantation* 2006; 82: 441.
11. Ohashi K, Park F, Kay MA. Hepatocyte transplantation: Clinical and experimental application. *J Mol Med* 2001; 79: 617.
12. Dhawan A, Mitry RR, Hughes RD, et al. Hepatocyte transplantation for inherited factor VII deficiency. *Transplantation* 2004; 78: 1812.

13. Ohashi K, Waugh JM, Dake MD, et al. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. *Hepatology* 2005; 41: 132.
14. Berry MN, Friend DS. High-yield preparation of isolated rat liver parenchymal cells: A biochemical and fine structural study. *J Cell Biol* 1969; 43: 506.
15. Ohashi K, Kay MA, Kuge H, et al. Heterotopically transplanted hepatocyte survival depends on extracellular matrix components. *Transplant Proc* 2005; 37: 4587.
16. Ohashi K, Kay MA, Yokoyama T, et al. Stability and repeat regeneration potential of the engineered liver tissues under the kidney capsule in mice. *Cell Transplant* 2005; 14: 621.
17. Lin HF, Maeda N, Smithies O, et al. A coagulation factor IX-deficient mouse model for human hemophilia B. *Blood* 1997; 90: 3962.
18. Snyder RO, Miao C, Meuse L, et al. Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. *Nat Med* 1999; 5: 64.
19. Schneider A, Attaran M, Gratz KF, et al. Intraportal infusion of 99mtechnetium-macro-aggregrated albumin particles and hepatocytes in rabbits: Assessment of shunting and portal hemodynamic changes. *Transplantation* 2003; 75: 296.
20. Kasper CK, Pool JG. Letter: Measurement of mild factor VIII inhibitors in Bethesda units. *Thromb Diath Haemorrh* 1975; 34: 875.
21. Arruda VR, Hagstrom JN, Deitch J, et al. Posttranslational modifications of recombinant myotube-synthesized human factor IX. *Blood* 2001; 97: 130.
22. Kokudo N, Ohashi K, Takahashi S, et al. Effect of 70% hepatectomy on DNA synthesis in rat hepatocyte isograft into the spleen. *Transplant Proc* 1994; 26: 3464.
23. Zhang H, Miescher-Clemens E, Drugas G, et al. Intrahepatic hepatocyte transplantation following subtotal hepatectomy in the recipient: A possible model in the treatment of hepatic enzyme deficiency. *J Pediatr Surg* 1992; 27: 312.
24. Ohashi K, Marion PL, Nakai H, et al. Sustained survival of human hepatocytes in mice: A model for in vivo infection with human hepatitis B and hepatitis delta viruses. *Nat Med* 2000; 6: 327.
25. Yokoyama T, Ohashi K, Kuge H, et al. In vivo engineering of metabolically active hepatic tissues in a neovascularized subcutaneous cavity. *Am J Transplant* 2006; 6: 50.
26. Ohashi K, Yokoyama T, Yamato M, et al. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. *Nat Med* 2007; 13: 880.

Biochemical and Biophysical Research Communications 374 (2008) 106–110

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reference gene selection for real-time RT-PCR in regenerating mouse livers

Kohei Tatsumi [a], Kazuo Ohashi [b,*], Sanae Taminishi [a], Teruo Okano [b], Akira Yoshioka [a], Midori Shima [a]

[a] Department of Pediatrics, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
[b] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

ARTICLE INFO

Article history:
Received 19 June 2008
Available online 3 July 2008

Keywords:
Liver regeneration
Hepatocyte proliferation
Partial hepatectomy
Housekeeping gene
Real-time RT-PCR
GeNorm
NormFinder
Mouse
PPIA
TBP

ABSTRACT

The liver has an intrinsic ability to undergo active proliferation and recover functional liver mass in response to an injury response. This regenerative process involves a complex yet well orchestrated change in the gene expression profile. To produce accurate and reliable gene expression of target genes during various stages of liver regeneration, the determination of internal control housekeeping genes (HKGs) those are uniformly expressed is required. In the present study, the gene expression of 8 commonly used HKGs, including GAPDH, ACTB, HPRT1, GUSB, PPIA, TBP, TFRC, and RPL4, were studied using mouse livers that were quiescent and actively regenerating induced by partial hepatectomy. The amplification of the HKGs was statistically analyzed by two different mathematical algorithms, geNorm and NormFinder. Using this method, PPIA and TBP gene expression found to be relatively stable regardless of the stages of liver regeneration and would be ideal for normalization to target gene expression.

The process of liver regeneration is a crucial intrinsic event by which the liver is able to recover from a loss of functional hepatic mass following injuries due to either surgical resection, or toxic, chemical or viral-based challenges [1,2]. The molecular events that are involved in the liver regeneration process are very complex, and the altered gene expressions ultimately orchestrate the integration of these distinct pathways to promote the regenerative biological response. Multiple studies have elucidated potential mechanistic pathways that may be involved in the process of liver regeneration, but many aspects of this phenomenon in terms of its gene expression profiles and its associated functional phenotypes remain to be further elucidated.

Among currently available methods to analyze gene expression profiles, reverse transcriptase coupled to real-time polymerase chain reaction (real-time RT-PCR) has recently been shown to be more efficient and reliable compared to the other methods [3]. To accurately quantify gene expression, one method is to normalize the target unknown gene expression level to an endogenously expressed reference gene(s), which are frequently housekeeping genes (HKGs). The ideal HKGs should be expressed at a constant level regardless of the liver regeneration status, otherwise the normalization using particular HKGs will lead to erroneous gene expression profiles of the target gene of interest. Due to the rapid and differential phenotypic changes during liver regeneration process, it is crucial to determine HKGs that remain unaltered throughout the regenerative time period, which generally is terminated by day 5 [2].

Statistical algorithms such as geNorm [4] and NormFinder [5] have been previously developed to evaluate the suitability of reference HKGs for use as a normalization marker following quantitative RT-PCR data in a given set of biological samples. Using these methods of statistical analysis, various HKGs have been recently assessed to determine their level of expression under specific conditions [6,7].

In the present study, we induced liver regeneration in mice using the most commonly used experimental procedure to promote hepatocyte proliferation, known as the 2/3 partial hepatectomy (PHx) [8]. Using this model of liver regeneration, the gene expression levels of 8 commonly used HKGs (GAPDH, ACTB, HPRT1, GUSB, PPIA, TBP, TFRC, and RPL4) and 2 liver-expressing target genes were investigated in quiescent and actively proliferating livers at different time points following PHx. The RT-PCR results were subsequently analyzed by two computer programs, geNorm and NormFinder, to select the best candidate reference gene during the liver regenerative process. To our knowledge, the present study describes the first systematic study to examine a series of HKGs to determine their utility as reference markers to evaluate the expression levels of target genes in regenerating mouse livers.

* Corresponding author. Fax: +81 3 3359 6046.
E-mail address: ohashi@abmes.twmu.ac.jp (K. Ohashi).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.06.103

Materials and methods

Animals. A total of 25 female wild-type C57BL/6 mice, 10–12 weeks old, were used in this study. Experimental protocols were developed in accordance with the guidelines outlined by our local animal committee located at Nara Medical University. Mice were placed in cages within a temperature-controlled room having a 12-h light/dark cycle (8:00 AM lights on/8:00 PM lights off).

2/3 Partial hepatectomy (PHx). The stimuli for liver regeneration was induced by a 2/3 partial hepatectomy on the C57BL6 wild-type mice ($n = 25$) as described previously [8–11]. The hepatectomy was performed at within a specified time window (between 8:00 and 10:00 AM) in order to minimize the circadian rhythm variations that may influence the speed and peak of the regenerative activity [12]. The liver lobes that were removed from each mouse at the time of hepatectomy were used as control liver samples in the quiescent state ($n = 25$). The mice were sacrificed at different time points (1, 2, and 5 days after hepatectomy; $n = 9$, 8, and 8, respectively) at which time the remainder of the liver lobes were harvested to determine the gene expression at different temporal points of liver regeneration.

RNA isolation and quality controls. Total RNA was extracted from each liver sample using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). DNaseI was used to digest and remove genomic DNA contamination. The RNA concentration of each sample was measured at a wavelength of 260 nm (A260). The purity of extracted total RNA was determined by the A260/A280 ratio. The real-time RT-PCR analyses were only performed on samples that had A260/A280 ratios between 1.9 and 2.1. The integrity of RNA samples was confirmed by electrophoresis on a 1% agarose gel.

Reverse transcription (RT) coupled quantitative real-time PCR. Total RNA (1 μg) was reverse-transcribed using oligo $d(T)_{16}$ primers and Omniscript RT Kit (QIAGEN). First-strand cDNA samples were subjected to quantitative PCR amplification using the PRISM 7700 Sequence Detector (Applied Biosystems Japan, Tokyo, Japan). Each of the liver cDNAs was determined for the expression levels of 8 commonly used housekeeping genes (HKGs) and two target genes as shown in Table 1. TaqMan probes and primers were chosen from the TaqMan Gene Expression Assay (Applied Biosystems) (Table 1). All of the PCR cycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. For quantification of gene expression, the cDNAs derived from pooled normal mouse livers were used to generate the reference standard curves.

Statistical analysis and determination of appropriate HKGs by geNorm and NormFinder. Significant differences between the non-normalized gene expression levels of quiescent and regenerating liver samples were analyzed by two-tailed Mann–Whitney *U*-test using Excel with ystat2006 software (Igakutosyosyuppan, Tokyo, Japan). $P < 0.05$ was considered significant. For stability comparisons of candidate reference genes, two additional validation software programs, geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) [4], and NormFinder (http://www.mdl.dk/publicationsnormfinder.htm) [5] were used.

Results

RNA quality control

The mean A260/280 ratio of the extracted RNA of the 25 quiescent and 25 regenerating livers averaged 2.02 ± 0.09 (ranging from 1.95 to 2.10), reflecting the purity and protein-free nature of the RNA. The RNA integrity was characterized by the 28 S/18 S ratio on a 1% agarose gel, and the ratios of all samples were >1.0.

Non-normalized expression levels of candidate reference genes

Non-normalized gene expression levels of 8 candidate HKGs were quantified, and the data was reported as comparative ratio to the day 0 samples. As shown in Fig. 1, the expression levels of GAPDH, ACTB, GUSB, PPIA, and TFRC were significantly higher at days 1 and/or 2 after the hepatectomy, and tended to decrease back towards basal levels by day 5. The *P* values from day 1, 2, and 5 compared to the day 0 values are as follows: GAPDH: 0.007 (day 1), 0.195 (day 2), and 0.46 (day 5), ACTB: 0.053, 0.0004, and 0.649, GUSB: 0.158, 0.004, and 0.051, PPIA: 0.028, 0.0002, and 0.125, and TFRC: 0.758, 0.006, and 0.074. HPRT1 expression was also significantly upregulated at day 2, but the upregulated levels persisted through to day 5. The *P* values of day 1, 2, and 5 compared to the day 0 values were 0.065, 0.001, and 0.017, respectively. In marked contrast, expression levels of TBP and RPL4 genes did not show any significant differences between day 0 with the other days 1, 2, and 5; the *P* values were as follows: TBP: 0.644, 0.23, and 0.35; and RPL4: 0.073, 0.249, and 0.258, respectively. These indicated that the gene expression levels of TBP and RPL4 were constant during the liver regeneration regardless of the time point after partial hepatectomy.

Statistical validation of an appropriate reference gene by geNorm and NormFinder

To analyze and provide a rank order of the 8 candidate HKGs in the quiescent and regenerating liver samples, we utilized geNorm

Table 1
Description of housekeeping gene (HKG) and target gene primers used in the quantitative RT-PCR assay

Symbol	Gene name	Function	Assay ID	Amplicon length (bp)
Housekeeping genes				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	Mm99999915_g1	107
ACTB	Actin, beta	Cytoskeletal structural protein	Mm00607939_s1	115
HPRT1	Hypoxanthine phosphoribosyltransferase	Purine synthesis in salvage pathway	Mm03024075_m1	131
GUSB	Glucuronidase, beta	Glycoside hydrolases cleaving glucuronides	Mm00446953_m1	76
PPIA	Peptidylprolyl isomerase A	Catalyzing the *cis-trans* isomerization of proline imidic peptide bonds in oligopeptides and accelerating the folding of proteins	Mm02342430_g1	148
TBP	TATA box binding protein	General RNA polymerase II transcription factor	Mm00446973_m1	73
TFRC	Transferrin receptor	Carrier protein for transferrin	Mm00441941_m1	66
RPL4	Ribosomal protein L4	Component of the 60S subunit of ribosome	Mm00834993_g1	129
Target genes				
OTC	Ornithine carbamoyltransferase	Enzyme catalyzing the reaction of urea cycle	Mm00493267_m1	102
AAT	α1-antitrypsin	Glycoprotein functioning as serum trypsin inhibitor	Mm00522856_m1	58

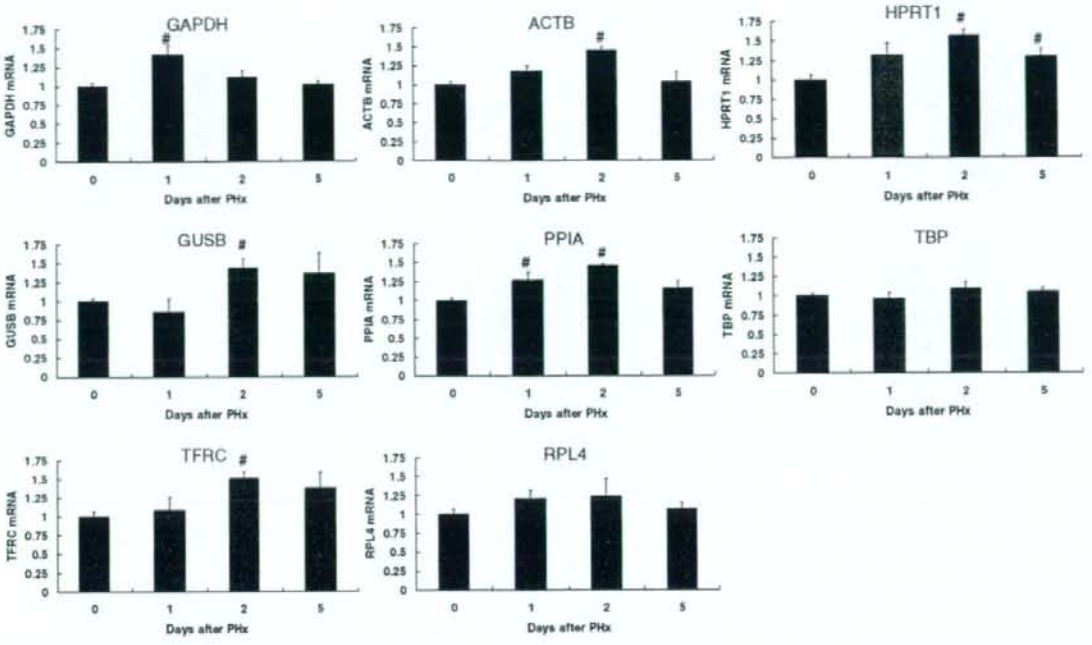

Fig. 1. The expression levels of 8 candidate HKGs in mouse livers. Non-normalized expression levels of the 8 HKGs of mouse livers under quiescent and regenerating status. The liver lobes removed at the time of hepatectomy were used as liver samples under quiescence status (day 0, $n = 25$). The remaining liver lobes obtained 1, 2, or 5 days after hepatectomy were used as samples to study the different stages of regeneration ($n = 9$, 8, and 8, respectively). The data was expressed as a comparative ratio to the day 0 samples, and represented as the mean ± SEM. $^{\#}P < 0.05$ vs day 0.

software. The geNorm is a statistical algorithm that was designed to determine the measure of stability (M) for all of the candidate genes based on the geometric averaging of multiple control genes, as well as the mean pairwise variation of a gene from all other control genes in a given set of samples [4].

The geNorm program relies on the principle that the expression ratio of two ideal internal control genes is identical in all of the samples regardless of the experimental condition. The genes with the lowest M values will be considered to have the most stable expression across time in the quiescent and regenerating livers obtained at days 1, 2, and 5 after the hepatectomy procedure. As a result, the ranking of gene expression stability value (M) of tested HKGs were as follows; GUSB > TFRC > RPL4 > HPRT1 > ACTB > GAPDH > PPIA, and TBP. This rank order data analysis indicated that PPIA and TBP were the most stable HKGs making them ideal for our quantitative analyses due to their lack of change during the liver regenerative time line (Fig. 2A).

In some cases, the normalization with two or more stable internal control reference genes may be required. Therefore, pairwise variations were calculated using geNorm for each data set to determine the optimal number of internal reference genes needed for normalization. In brief, the normalization factors (NF) were calculated for the most stable control genes (i.e., the lowest M value) and then other reference genes with the next lowest M values were added in a stepwise manner. Subsequently, pairwise variations of NF_n and NF_{n+1} were calculated, reflecting the effect of including additional ($n + 1$) genes [4]. From these analyses, two genes with most stable expression (TBP and PPIA) were found to be optimal for reliable normalization with a pairwise variation cut-off value of 0.15 (Fig. 2B). Below this cut-off value, there was no need to include an additional HKG, because the inclusion of a third gene had no significant effect on the pairwise variation.

Next, we used NormFinder, which was another algorithm to calculate the most stable HKGs in our set of 8 candidate HKGs [5]. The

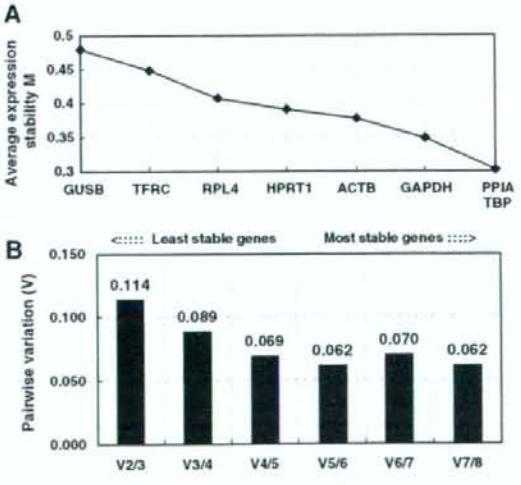

Fig. 2. (A) Average expression stability values (M) of 8 candidate housekeeping genes (HKGs). HKGs were graphed to illustrate the average gene expression stability (M) on the Y-axis, and its associated ranking from least to most stable expression (X-axis) as calculated by geNorm ($n = 50$ liver samples). Lower M value of average expression stability indicates more stable expression. (B) Determination of the optimal number of control genes for normalization. The optimal number of control genes for normalization was calculated on the basis of a pair-wise variation (V) analysis.

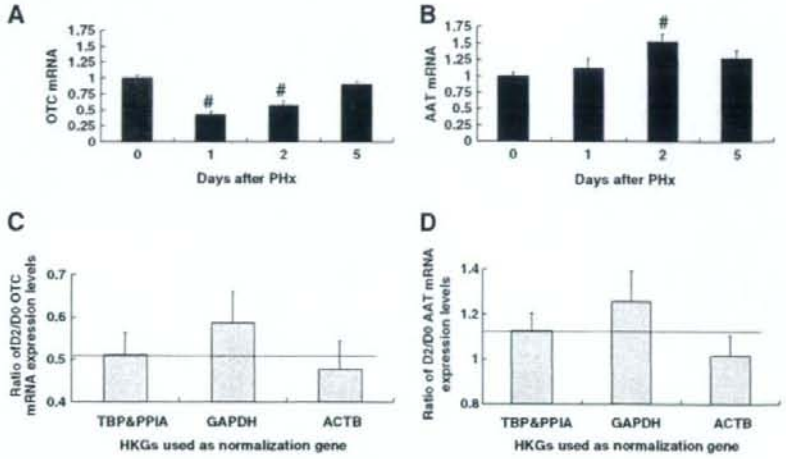

Fig. 3. Expression levels of two target genes, ornithine carbamoyltransferase (OTC) and α1-antitrypsin (AAT). (A and B) Non-normalized expression of two target genes transcribed in mouse livers during states of quiescence and active regeneration post-hepatectomy. The liver lobes removed at the time of hepatectomy were used as quiescent liver samples (day 0, $n = 25$). The remaining liver lobes were obtained 1, 2, or 5 days after hepatectomy and used as the proliferative liver samples ($n = 9$, 8, and 8, respectively). Data was expressed as a ratio to the day 0 liver samples and were represented as the mean ± SEM. $^{\#}P < 0.05$ vs day 0. (C and D) The gene expression levels of OTC and AAT were normalized to three different sets of HKGs (the geometric mean of PPIA and TBP, GAPDH, and ACTB). The normalized gene expression values were shown for the day 2 liver samples relative to the corresponding expression values at day 0 ($n = 8$). Data was represented as the mean ± SEM.

NormFinder was designed to calculate the stability by using the combined estimate of intra- and intergroup expression variations of the analyzed genes. The calculated stability values of the 8 candidate HKGs were as follows: GAPDH = 0.172; ACTB = 0.138; HPRT1 = 0.132; GUSB = 0.199; PPIA = 0.088; TBP = 0.118; TFRC = 0.167; and RPL4 = 0.142. Based on these values, the NormFinder program validated the findings with the geNorm algorithm, in which the most stable single gene was PPIA, and the best combination of the HKGs was PPIA and TBP.

Expression levels of target gene influenced by the choice of a normalization gene(s)

The expression profile of target genes can be markedly influenced depending on the choice of the normalization gene(s). The expression levels of the two target genes, ornithine carbamoyltransferase (OTC) and α1-antitrypsin (AAT), both of which are synthesized by liver, were assessed. We determined the non-normalized mRNA expression profile of OTC and AAT in the same liver samples used in the determination of the HKG expression profile. The expression of OTC genes was shown to be significantly suppressed 1 and 2 days after hepatectomy (Fig. 3A), whereas the AAT gene expression was upregulated 2 days after hepatectomy ($P < 0.05$ vs quiescent status) (Fig. 3B). The mRNA expression levels of the liver samples 2 days after hepatectomy were normalized against three different sets of HGK, specifically GAPDH, ACTB and the geometric mean of PPIA and TBP. Subsequently, the normalized day 2 values were divided by the normalized liver values obtained at day 0. As shown in Fig. 3C and D, though not significantly, both OTC and AAT mRNA levels that were normalized by the geometric mean of PPIA and TBP were lower than levels normalized by GAPDH, and higher than those normalized by ACTB. These results indicate that depending on which particular HKG was used in the normalization, the expression profile of the target gene can be influenced and produce different levels of gene expression.

Discussion

The appropriate choice of individual or a group of reference genes, in general endogenously expressed housekeeping genes (or HKGs), is critical in the quantification of gene expression profiles under different conditions, such as liver regeneration. In the present study, we examined 8 candidate HKGs that were selected in terms of their broad use in previously published studies using the liver and other type of tissues. The expression levels of the candidate HKG were assessed in quiescent or regenerating mouse livers at various stages of proliferation. The present results were based on geNorm and NormFinder analyses, which confirmed that the expression levels of the PPIA and TBP reference genes were the most stable among the 8 candidates regardless of the cell cycle status of the livers.

In the first 24 h following a partial hepatectomy in mice, hepatocyte DNA synthesis is initiated. Over the next 24 h period, the DNA synthesis phase become synchronized and peaks, and then slowly returns back to normal levels by day 5 after the hepatectomy. At day 5, the remnant liver lobes have undergone sufficient hyperplasia to return the liver weight back to its original pre-hepatectomy weight [2,13]. This rapid process from initiation to completion of the regeneration event prompted us to assess a panel of HKGs using liver samples harvested at day 1, 2, and 5 after the hepatectomy procedure.

In this present study, mRNA expression levels were upregulated in 6 out of 8 HKGs analyzed during the active phase of liver regeneration (days 1 and/or 2). The upregulation of these particular HKGs demonstrated that they likely play a vital role in the events related with DNA synthesis, cell division and hepatic growth. As an example, GAPDH and ACTB genes, which have been widely applied for the normalization of gene expression analysis of various types of tissues, showed significant increases in the mRNA expression at day 1 and/or 2 after hepatectomy (Fig. 1). The temporal upregulation of these two HKGs in the liver regeneration process were consistent with previous reports [14–16]. We have recently

showed that human hepatocytes undergoing active proliferation following transplantation into mouse liver exhibited higher GAPDH mRNA expression compared to normal human livers [17]. These data taken together, suggest that these two genes may not be appropriate reference genes to normalize target gene expression, particularly in the context of liver regeneration. Therefore, the identification of suitable internal reference genes that are not affected by a given experimental condition is critically important to accurately analyze expression levels of target genes. To address this problem, we utilized two different algorithms, geNorm and Normfinder [4,5], to select an optimal reference gene from a panel of candidate HKGs in quiescent and regenerating mouse livers. As a result, we found that the PPIA and TBP mRNA were most stably expressed, and both programs concluded that a combination of the two genes were the most appropriate for normalization of unknown target genes to determine their expression profiles in liver regeneration (Fig. 1). From examining the non-normalized RPL4 gene expression (Fig. 1), the values were found to be stable regardless of the time point. However, analyzing the expression data in a comprehensive manner using the two software programs demonstrated that the RPL4 reference gene was not stable. This is one example in which non-normalized data is only one method to demonstrate the potential reliability as a reference gene, but that other comparative analyses are needed, such as the geNorm and NormFinder, to further validate the suitability of selecting appropriate HKGs.

PPIA (peptidylprolyl isomerase A), also called as cyclophilin A, forms a ternary complex with cyclosporin A and the calcium-calmodulin-activated serine/threonine-specific protein phosphatase calcineurin [18]. This gene has been recently reported to be preferred over other commonly used HKGs following its analysis in various cell lines and tissues [19]. The other stably expressed reference gene, TBP (also known as TATA box binding protein), is a protein required by all three eukaryotic RNA polymerases to correctly initiate the transcription of ribosomal, messenger, small nuclear and transfer RNAs [20]. The usefulness of TBP gene has also been validated in other sets of experimental conditions [21]. Although there is a strong possibility that other more suitable reference genes other than the ones presently analyzed in our study, we have confirmed that PPIA and TBP genes gave us reliable and stable gene expression compared to other more commonly used HKGs, including ACTB or GAPDH (Fig. 3C and D).

The elucidation of liver regeneration events are further supported by recent studies in which hepatocyte-based approaches were developed, including liver tissue engineering [10,11,22], hepatocyte transplantation [23], hepatocyte propagation [17], and hepatic differentiation from stem cells [24]. Taken together, the results from the current study where we validated merit of normalizing target gene expression with the combined expression values of PPIA and TBP genes should serve as a viable method to accurately quantify gene expression profiles related with liver regeneration.

Acknowledgments

The authors thank Dr. Frank Park (Medical College of Wisconsin) for his critical reading of the manuscript. This study was supported by grants for AIDS Research from the Ministry of Health, Labor and Welfare of Japan (A.Y.), Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan (K.O. and T.O.), Novartis Foundation Japan (K.O.), and Bayer Hemophilia Award Program (K.O.).

References

[1] N. Fausto, J.S. Campbell, K.J. Riehle, Liver regeneration, Hepatology 43 (2006) S45–S53.
[2] G.K. Michalopoulos, Liver regeneration, J. Cell. Physiol. 213 (2007) 286–300.
[3] S.A. Bustin, Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, J. Mol. Endocrinol. 29 (2002) 23–39.
[4] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002). RESEARCH0034.
[5] C.L. Andersen, J.L. Jensen, T.F. Orntoft, Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets, Cancer Res. 64 (2004) 5245–5250.
[6] Q. Gao, X.Y. Wang, J. Fan, S.J. Qiu, J. Zhou, Y.H. Shi, Y.S. Xiao, Y. Xu, X.W. Huang, J. Sun, Selection of reference genes for real-time PCR in human hepatocellular carcinoma tissues, J. Cancer Res. Clin. Oncol. (2008).
[7] M. Jain, A. Nijhawan, A.K. Tyagi, J.P. Khurana, Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR, Biochem. Biophys. Res. Commun. 345 (2006) 646–651.
[8] A.K. Greene, M. Puder, Partial hepatectomy in the mouse: technique and perioperative management, J. Invest. Surg. 16 (2003) 99–102.
[9] K. Ohashi, M.A. Kay, T. Yokoyama, H. Kuge, H. Kanehiro, M. Hisanaga, S. Ko, Y. Nakajima, Stability and repeat regeneration potential of the engineered liver tissues under the kidney capsule in mice, Cell Transplant. 14 (2005) 621–627.
[10] K. Ohashi, J.M. Waugh, M.D. Dake, T. Yokoyama, H. Kuge, Y. Nakajima, M. Yamanouchi, H. Naka, A. Yoshioka, M.A. Kay, Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases, Hepatology 41 (2005) 132–140.
[11] K. Ohashi, T. Yokoyama, M. Yamato, H. Kuge, H. Kanehiro, M. Tsutsumi, T. Amanuma, H. Iwata, J. Yang, T. Okano, Y. Nakajima, Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets, Nat. Med. 13 (2007) 880–885.
[12] T. Matsuo, S. Yamaguchi, S. Mitsui, A. Emi, F. Shimoda, H. Okamura, Control mechanism of the circadian clock for timing of cell division in vivo, Science 302 (2003) 255–259.
[13] K. Ohashi, F. Park, M.A. Kay, Role of hepatocyte direct hyperplasia in lentivirus-mediated liver transduction in vivo, Hum. Gene Ther. 13 (2002) 653–663.
[14] Y. Fukuhara, A. Hirasawa, X.K. Li, M. Kawasaki, M. Fujino, N. Funeshima, S. Katsuma, S. Shiojima, M. Yamada, T. Okuyama, S. Suzuki, G. Tsujimoto, Gene expression profile in the regenerating rat liver after partial hepatectomy, J. Hepatol. 38 (2003) 784–792.
[15] Y. Ito, H. Hayashi, M. Taira, M. Tatibana, Y. Tabata, K. Isono, Depression of liver-specific gene expression in regenerating rat liver: a putative cause for liver dysfunction after hepatectomy, J. Surg. Res. 51 (1991) 143–147.
[16] Y. Kurumiya, K. Nozawa, K. Sakaguchi, M. Nagino, Y. Nimura, S. Yoshida, Differential suppression of liver-specific genes in regenerating rat liver induced by extended hepatectomy, J. Hepatol. 32 (2000) 636–644.
[17] K. Tatsumi, K. Ohashi, M. Kataoka, C. Tateno, M. Shibata, H. Naka, M. Shima, M. Hisanaga, H. Kanehiro, T. Okano, K. Yoshizato, Y. Nakajima, A. Yoshioka, Successful in vivo propagation of factor IX-producing hepatocytes in mice. Potential for cell-based therapy in haemophilia B, Thromb. Haemost. 99 (2008) 883–891.
[18] P. Wang, J. Heitman, The cyclophilins, Genome Biol. 6 (2005) 226.
[19] M. Nishimura, T. Nikawa, Y. Kawano, M. Nakayama, M. Ikeda, Effects of dimethyl sulfoxide and dexamethasone on mRNA expression of housekeeping genes in cultures of C2C12 myotubes, Biochem. Biophys. Res. Commun. 367 (2008) 603–608.
[20] S.K. Burley, The TATA box binding protein, Curr. Opin. Struct. Biol. 6 (1996) 69–75.
[21] M. Jung, A. Ramankulov, J. Roigas, M. Johannsen, M. Ringsdorf, G. Kristiansen, K. Jung, In search of suitable reference genes for gene expression studies of human renal cell carcinoma by real-time PCR, BMC Mol. Biol. 8 (2007) 47.
[22] K. Ohashi, P.L. Marion, H. Nakai, L. Meuse, J.M. Cullen, B.B. Bordier, R. Schwall, H.B. Greenberg, J.S. Glenn, M.A. Kay, Sustained survival of human hepatocytes in mice. A model for in vivo infection with human hepatitis B and hepatitis delta viruses, Nat. Med. 6 (2000) 327–331.
[23] K. Tatsumi, K. Ohashi, M. Shima, Y. Nakajima, T. Okano, A. Yoshioka, Therapeutic effects of hepatocyte transplantation on hemophilia B, Transplantation (2008).
[24] S. Kasuda, A. Kubo, Y. Sakurai, S. Irion, K. Ohashi, K. Tatsumi, Y. Nakajima, Y. Saito, K. Hatake, S.W. Pipe, M. Shima, A. Yoshioka, Establishment of embryonic stem cells secreting human factor VIII for cell-based treatment of hemophilia A, J. Thromb. Haemost. (2008).

Journal of Thrombosis and Haemostasis, **6**: 1352–1359 DOI: 10.1111/j.1538-7836.2008.03022.x

ORIGINAL ARTICLE

Establishment of embryonic stem cells secreting human factor VIII for cell-based treatment of hemophilia A

S. KASUDA*†‡, A. KUBO§, Y. SAKURAI*, S. IRION¶, K. OHASHI**[1], K. TATSUMI*, Y. NAKAJIMA**, Y. SAITO§, K. HATAKE†, S. W. PIPE‡‡, M. SHIMA* and A. YOSHIOKA*

*Department of Paediatrics, Nara Medical University, Kashihara, Nara; †Department of Legal Medicine, Nara Medical University, Kashihara, Nara; ‡Department of Legal Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo; §First Department of Internal Medicine, Nara Medical University, Kashihara, Nara, Japan; ¶Department of Gene and Cell Medicine, Mount Sinai School of Medicine, New York, NY, USA; **Department of Surgery, Nara Medical University, Kashihara, Nara, Japan; and ‡‡Department of Pediatrics, University of Michigan Medical Center, Ann Arbor, MI, USA

To cite this article: Kasuda S, Kubo A, Sakurai Y, Irion S, Ohashi K, Tatsumi K, Nakajima Y, Saito Y, Hatake K, Pipe SW, Shima M, Yoshioka A. Establishment of embryonic stem cells secreting human factor VIII for cell-based treatment of hemophilia A. *J Thromb Haemost* 2008; **6**: 1352–9.

Summary. *Background:* Hemophilia A is an X-chromosome-linked recessive bleeding disorder resulting from an *F8* gene abnormality. Although various gene therapies have been attempted with the aim of eliminating the need for factor VIII replacement therapy, obstacles to their clinical application remain. *Objectives:* We evaluated whether embryonic stem (ES) cells with a tetracycline-inducible system could secrete human FVIII. *Methods and results:* We found that embryoid bodies (EBs) developed under conditions promoting liver differentiation efficiently secreted human FVIII after doxycycline induction. Moreover, use of a B-domain variant *F8* cDNA (226aa/N6) dramatically enhanced FVIII secretion. Sorting based on green fluorescent protein (GFP)–brachyury (Bry) and c-kit revealed that GFP–Bry^+/$c\text{-}kit^+$ cells during EB differentiation with serum contain an endoderm progenitor population. When GFP–Bry^+/$c\text{-}kit^+$ cells were cultured under the liver cell-promoting conditions, these cells secreted FVIII more efficiently than other populations tested. *Conclusion:* Our findings suggest the potential for future development of an effective ES cell-based approach to treating hemophilia A.

Keywords: cell-based therapy, embryonic stem cells, factor VIII, hemophilia A.

Correspondence: Atsushi Kubo, First Department of Internal Medicine, Nara Medical University, 840 Shijo, Kashihara, Nara 634-8522, Japan.
Tel.: +81 744 22 3051 ext. 3411; fax: +81 744 22 9726.
E-mail: akubo@naramed-u.ac.jp

[1]Present address: Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan

Received 24 October 2007, accepted 25 April 2008

Introduction

Hemophilia A is an X-chromosome-linked recessive bleeding disorder resulting from an inversion or mutation within the *F8* gene, and is the most common of the congenital bleeding disorders [1]. The clinical severity of hemophilia A correlates closely with circulating levels of factor VIII (FVIII) protein. Current standard therapy for hemophilia A patients is replacement therapy with intravenous infusion of plasma-derived or recombinant FVIII concentrates [2]. However, the half-life of infused FVIII is short (10–12 h), and the cost of the frequent infusions necessary to maintain adequate plasma levels of FVIII is extremely high. Consequently, the development of a novel therapy leading to constitutive supply of FVIII is much desired in the next stage of the treatment of hemophilia. For that reason, gene therapy for hemophilia has received a great deal of attention [3]. Constant and sustained FVIII synthesis mediated by gene therapy in patients would obviate the risk of spontaneous bleeding without the need for repeated FVIII infusions. Although the gene therapy approach has shown promise in a mouse model [4], some drawbacks, such as hepatic damage or viral contamination in the non-motile sperm, have been reported [5,6].

As another approach, orthotopic liver transplantation (OLT) has also been attempted for the treatment of hemophilia [7]. Moreover, recent studies have shown that transplantation of hepatocytes [8] or sinusoidal endothelial cells [9] corrects the hemophilia A phenotype in mice, suggesting that cell-based therapy using primary cultured cells may be a useful approach to treating hemophilia. However, a disadvantage of these therapies is that there is a shortage of donors for transplantation or cell isolation.

Given the limitations of all the aforementioned therapies, we evaluated the potential of a cell-based therapy that makes use of embryonic stem (ES) cells as the source of active human FVIII. ES cells retain their totipotential capacity when

maintained on mouse embryonic feeder (MEF) cells and are able to spontaneously differentiate and generate various lineages via the embryoid body (EB) stage. We hypothesized that ES cell-based therapy would have unique characteristics that would enable us to overcome the problems associated with gene therapy or primary cultured cell transplantation. First, ES cells can provide a cell source with unlimited expansion capacity, thereby overcoming the shortage of donors for OLT or primary cultured cell transplantation. Second, hepatic damage or the contamination in the non-motile sperm fraction by viral vectors would be avoided, as there is no virus present.

In this study, to induce the human *F8* gene in ES cells, we used an ES cell line (Ainv18) that enables the inducible expression of the *F8* gene under the control of a tet-inducible promoter [10]. Although Ainv18 ES cells have been used previously for functional analysis of the transcriptional factors HoxB4 and Hex [10,11], we used them for synthesis of a secretable protein. Together, these advantageous features could make ES cell-based therapy an effective approach to the treatment of hemophilia A. Our aim in the present study, therefore, was to establish an ES cell line capable of doxycycline (Dox)-inducible *F8* gene expression and to determine the most suitable differentiation conditions for secretion of FVIII. We show that ES cells can secrete FVIII with antigen and coagulant activity, suggesting that ES cell-based therapy may be a potentially useful approach to treating hemophilia A.

Materials and methods

Growth and differentiation of ES cells

The cDNA construct harboring the full-length human wild-type (WT)-*F8* was described previously [12], as were the B-domain-deleted (BDD)-*F8* and 226aa/N6 cDNAs [13]. Ainv18 ES cells (a kind gift from M. Kyba and G. Q. Daley) were transfected with the WT-*F8*-plox, BDD-*F8*-plox or 226aa/N6-plox targeting plasmids by electroporation, yielding tet-WT-*F8*, tet-BDD-*F8* and tet-226aa/N6 ES cells, after which the transfectants were selected with G418, as described previously [11]. Green fluorescent protein (GFP)–brachyury (Bry) Ainv18 ES cells (S. Irion *et al.*, unpublished data) were established by targeting GFP to the Bry locus in Ainv18 ES cells [14].

ES cells were maintained on MEF cells and were passaged twice on gelatin-coated dishes before EB formation, as previously described [15]. To generate EBs, ES cells were dissociated to a single cell suspension with 0.25% trypsin/EDTA and cultured at various concentrations ($1–8 \times 10^3$ cells mL^{-1}) in 60-mm Petri-grade dishes in serum-containing differentiation medium [Iscoves' modified Dulbecco's medium (IMDM) supplemented with penicillin–streptomycin, 2 mM glutamine (Gibco/BRL, Grand Island, NY, USA), 0.5 mM ascorbic acid (Sigma-Aldrich, St Louis, MO, USA), 0.45 mM monothioglycerol (MTG; Sigma-Aldrich), 15% fetal bovine serum (FBS; Vitromex, Geilenkirchen, Germany), 5% protein-free hybridoma medium (Gibco/BRL) and 200 μg mL^{-1} transferrin (Boehringer Mannheim, Indianapolis, IN, USA)]. Cultures were maintained in a humidified chamber in a 5% CO_2/air mixture at 37 °C.

The experimental protocol is depicted schematically in Fig. 1A. When EBs were cultured in differentiation medium for 6 days, EBs differentiated into the mesodermal lineage, which includes mainly hematopoietic and endothelial cell populations (hematopoietic-like EBs). For liver differentiation, EBs were cultured in differentiation medium for 3 days and then transferred to serum replacement (SR) medium [IMDM supplemented with 15% knockout SR (Gibco/BRL), penicillin–streptomycin, 2 mM glutamine, 0.5 mM ascorbic acid, 0.45 mM MTG] and cultured for an additional 7 days. On day 10, the EBs were harvested and replated in 12-well tissue culture dishes coated with Matrigel (Becton Dickenson, San Jose, CA, USA) in IMDM with 15% FBS and 1 μM dexamethasone (Dex; Sigma-Aldrich), which led to the development of liver-like EBs [15].

Undifferentiated ES cells were passaged twice on gelatin. Hematopoietic-like EBs cultured with serum for 6 days were replated on 12-well culture dishes coated with Matrigel in IMDM with 15% FBS and 1 μM Dex for 2 days. Undifferentiated ES cells, day 8 hematopoietic-like EBs and day 13 liver-like EBs were stimulated with Dox for 2 days before assay. For *in vitro* assay of FVIII, the culture media were replaced with 500 μL of serum-free IMDM medium containing 5 mg mL^{-1} bovine serum albumin (Calbiochem, San Diego, CA, USA) [16] with or without human FVIII-free von Willebrand factor (VWF; Haematologic Technologies, Essex Junction, VT, USA). Twenty-four hours later, the supernatant and cell samples were harvested for determination of FVIII activity (FVIII:C) and FVIII antigen (FVIII:Ag) or protein levels.

Gene expression

For gene-specific reverse transcription polymerase chain reaction (RT-PCR), total RNA was extracted using RNeasy mini-kits and treated with RNase-free DNase (Qiagen, Valencia, CA, USA). One microgram of total RNA was reverse-transcribed into cDNA using a Superscript RT kit (Invitrogen, Carlsbad, CA, USA) with random hexamers. PCR was carried out using Taq polymerase (Takara Bio, Shiga, Japan) in PCR buffer, 2.5 mM $MgCl_2$, and 0.2 mM dNTPs. The primers for human specific *F8* were 5′-AGAGTTCCAAGCCTCCAACA-3′ (sense) and 5′-TAGACCTGGGTTTTCCATCG-3′ (antisense). The cycling protocol entailed one cycle of 94 °C for 5 min, followed by 25–35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s and elongation at 72 °C for 1 min, and a final incubation at 72 °C for 7 min. Oligonucleotides for *Rex1*, *Gata1*, *Albumin1* (*Alb*), *transthyretin* (*Ttr*), *tyrosine aminotransferase* (*Tat*), *α-fetoprotein* (*Afp*), *Foxa2*, *Sox17*, *Cereberus*, *E-cadherin* (*E-cad*), *Hex* and *β-actin* have been previously described [15,17]. Quantitative real-time RT-PCR analysis was performed with an Applied Biosystems Prism 7700 Sequence Detection System using TaqMan® universal PCR master mix according to the manufacturer's specifications (Applied Biosystems, Foster City, CA, USA).

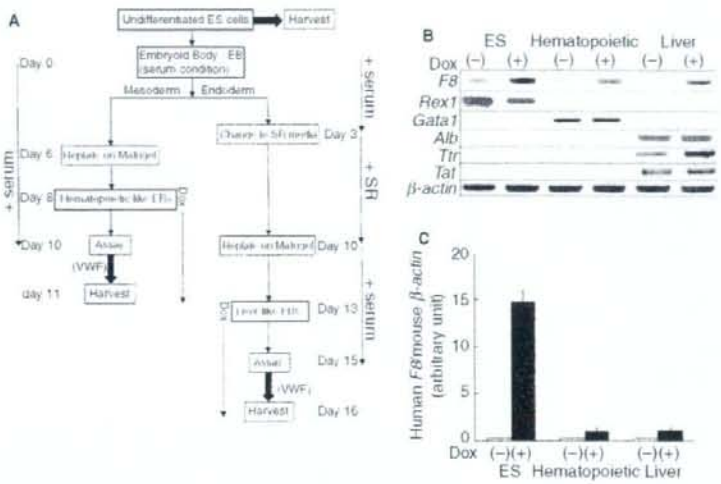

Fig. 1. Expressions of WT-*F8* mRNA by doxycycline (Dox) stimulation in undifferentiated embryonic stem (ES) cells, hematopoietic-like embryoid bodies (EBs) and liver-like EBs. (A) Schema of the experimental protocol. (B) Reverse transcription polymerase chain reaction (RT-PCR) analysis of variable marker genes in undifferentiated tet-WT-*F8* ES cells, hematopoietic-like EBs and liver-like EBs with or without Dox induction (1 μg mL^{-1}). (C) Real-time PCR analysis of *F8* mRNA levels in undifferentiated tet-WT-*F8* ES cells, hematopoietic-like EBs and liver-like EBs with or without Dox induction (1 μg mL^{-1}). The data presented are means of three independent experiments; the error bars represent the SEM. VWF, von Willebrand factor; SR, serum replacement.

The TaqMan probes and primers for human *F8* (assay identification number Hs00240767) and mouse *F8* (assay identification number Mm00433174) were assay-on-demand gene expression products (Applied Biosystems). The mouse *β-actin* gene (assay identification number Mm00607939) was used as an endogenous control.

FVIII assay

FVIII:C was measured in a one-stage activated partial thromboplastin time (APTT) clotting assay in a coagulometer (KC10A; Amelung, Lemgo, Germany) using human FVIII-deficient plasma (George King Biomedical, Overland Park, KS, USA). Activated partial thromboplastin and $CaCl_2$ were purchased from bioMerieux (Durham, NC, USA). FVIII:Ag was quantified using human FVIII-specific enzyme-linked immunosorbent assay (ELISA) kits (FVIII:C-EIA, Affinity Biologicals, Ancaster, ON, Canada), according to the manufacturer's instructions. These ELISA kits employ FVIII light chain specific antibody, and is the same kits used for 226aa/N6 detection previously [13]. For measurement of both FVIII:C and FVIII:Ag, a standard curve was generated using normal human plasma (Coagtrol N; Sysmex, Kobe, Japan) in serial doubling dilutions (1 : 10 to 1 : 1280) in 0.05 M imidazole saline buffer. Each supernatant sample was applied to these assays without dilution rather than 10× dilution. Therefore, FVIII:C and FVIII:Ag levels of culture supernatant samples should be considered as 1/10 of the raw data. We calculated FVIII:Ag levels in normal human plasma as 1 nM. The detection limits of the FVIII:C and FVIII:Ag assays were 10 mIU mL^{-1} and 10 pM, respectively. The attached cell samples in each well were also harvested to determine the amount of protein by a BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Although these types of data are typically signified in terms of cell number, it is very difficult to count cell numbers in liver-like EBs, due to formation of tight aggregates. In order to adjust secretion levels from the equal protein levels of EBs, FVIII:C and FVIII:Ag levels in the supernatant of each well were adjusted by protein amount of attached cells in the same well. Data are shown as 'not detected' when the raw data for FVIII:C and FVIII:Ag were under the detection limit of the assays (10 mIU mL^{-1} and 10 pM, respectively).

Cell sorting

Day 3.5 EBs were dissociated with trypsin–EDTA, stained with anti-mouse c-kit-phycoerythrin (BD PharMingen, San Diego, CA, USA) in IMDM supplemented with 5% FBS, and sorted in a FACS Aria cell sorter (Becton Dickinson). After sorting, the cells were reaggregated in SR medium and cultured using the liver differentiation protocol.

Results

Tet-WT-F8 ES cells secrete active human FVIII protein

Using the Ainv18 ES cell line, we established ES cells in which the *F8* gene was induced by the tetracycline analog Dox (tet-WT-*F8* ES cells). Tet-WT-*F8* ES cells were cultured such that

we were able to obtain three different cell types: undifferentiated ES cells, enriched hematopoietic EBs (hematopoietic-like EBs), and enriched liver EBs (liver-like EBs). RT-PCR analysis revealed that tet-WT-*F8* ES cells were well differentiated under hematopoietic cell-promoting conditions (hematopoietic conditions) or liver cell-promoting conditions (liver conditions; Fig. 1B), which is consistent with earlier findings [15]. *Rex1* and *Gata1* are marker genes for undifferentiated ES cells and hematopoietic cells, respectively. *Alb*, *Ttr* and *Tat* are marker genes for liver cells. Recently, we further confirmed that liver-like EBs also secreted albumin and transferrin (A. Kubo, unpublished data). Addition of Dox (1 μg mL^{-1}) to the culture medium successfully upregulated *F8* mRNA expression under all three differentiation conditions (Fig. 1B). We also quantitatively analyzed mRNA expression by real-time PCR (Fig. 1C). Interestingly, *F8* mRNA levels in undifferentiated ES cells were much higher than those in hematopoietic-like EBs and liver-like EBs. The reason for this is currently unclear. However, we deduce that gene induction by Dox may be more effective in undifferentiated ES cells than in the other differentiated EBs because of the three-dimensional structure of EBs. On the other hand, *F8* mRNA expression levels of the two cell types were found to be identical. Mouse *F8* mRNA was not induced in liver-like EBs (data not shown). Among the different cell types, FVIII:C and FVIII:Ag were detected only in the supernatant from liver-like EBs; neither was detected with undifferentiated ES cells or hematopoietic-like EBs (Fig. 2A,B). Apparently, the differentiation conditions and the resulting cell types are critical to the production and secretion of FVIII, despite the mRNA levels induced by Dox. In the presence of 2.5 μg mL^{-1} of VWF, Dox-induced levels of both FVIII:C and FVIII:Ag were increased to about twice that seen in the absence of VWF (data not shown). Accordingly, VWF was added at a concentration of 2.5 μg mL^{-1} in subsequent experiments. We also assessed the effect of Dox concentrations on FVIII secretion. When liver-like EBs were stimulated with various concentrations of Dox, the level of *F8* mRNA increased in a dose-dependent manner with increasing

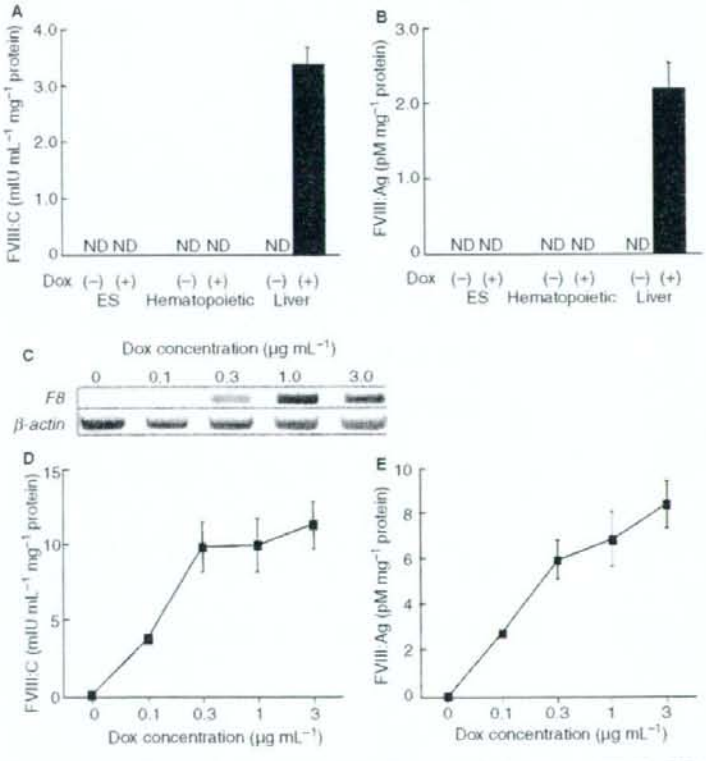

Fig. 2. FVIII:C and FVIII:Ag levels in undifferentiated embryonic stem (ES) cells, hematopoietic-like embryoid bodies (EBs) and liver-like EBs with or without doxycycline (Dox) stimulation. (A, B) FVIII:C (A) and FVIII:Ag (B) levels in media conditioned by cells cultured under the three differentiation conditions, with or without Dox induction (1 μg mL^{-1}). No von Willebrand factor (VWF) was added. (C) *F8* mRNA expression induced by the indicated concentrations of Dox from tet-WT-*F8* ES cells. (D, E) Secretion of FVIII:C (D) and FVIII:Ag (E) from tet-WT-*F8* ES cells induced by the indicated concentrations of Dox in the presence of 2.5 μg mL^{-1} VWF. The data presented are means of three independent experiments; the error bars represent the SEM. ND, not detected.

Dox concentrations (Fig. 2C), and there were corresponding increases in both FVIII:C and FVIII:Ag (Fig. 2D,E).

Tet-226aa/N6 ES cells secrete active FVIII more efficiently than tet-WT-F8 ES cells

Earlier reports showed that BDD-*F8* is more efficient than WT-*F8* for FVIII production, because higher mRNA levels are achieved [18,19]. In addition, Miao *et al.*[13] bioengineered a BDD-*F8* variant with 226 amino acids of the native *F8* B-domain that includes six asparagine-linked glycosylations (226aa/N6). They showed that COS-1 or CHO cells transfected with 226aa/N6 secrete active FVIII more efficiently than WT-*F8*- or BDD-*F8*-expressing cells. To evaluate these three *F8* types with respect to FVIII production and secretion, tet-WT-*F8* ES cells, tet-BDD-*F8* ES cells and tet-226aa/N6 ES cells were cultured under the liver conditions. Real-time PCR analysis showed that BDD-*F8* mRNA was expressed 2-fold higher than WT-*F8*, and 226aa/N6 mRNA levels were between those of WT-*F8* and BDD-*F8* (Fig. 3A). These results suggest that the length of the B-domain may affect the transcriptional levels of the *F8* gene.

FVIII secretion in liver-like EBs from tet-BDD-*F8* ES cells was about 1.5-fold higher than in those from tet-WT-*F8* ES cells (Fig. 3B,C). Furthermore, FVIII secretion in liver-like EBs from tet-226aa/N6 ES cells was about 6–10-fold higher than in those from tet-WT-*F8* ES cells (Fig. 3B,C). These results demonstrated that the construct of 226aa/N6 efficiently produced higher levels of *F8* regardless of transcriptional levels.

Comparison of FVIII secretion in population sorting based on the Bry/c-kit

Recently, Gouon-Evans *et al.* showed that activin can induce definitive endoderm in the absence of serum and that the GFP–Bry$^+$/c-kit$^+$ population was the definitive endoderm progenitor under this condition [20]. We tested whether the GFP–Bry$^+$/c-kit$^+$ population cultured in the presence of serum also contained endoderm progenitors, and which subpopulation gave rise to FVIII-secreting cells. Tet-226aa/N6 ES cells were differentiated for 3.5 days in the presence of serum, at which time the GFP–Bry$^+$ and c-kit$^+$ populations had been induced (Fig. 4A). On the basis of earlier studies [14,20], the GFP–Bry$^+$/c-kit$^-$ and GFP–Bry$^+$/c-kit$^+$ cell fractions were deemed to be mesoderm and endoderm, respectively. After the population was sorted and harvested for RNA isolation, RT-PCR showed that *Foxa2* and *Sox17*, which are normally expressed in endoderm, were expressed primarily in GFP–Bry$^+$/c-kit$^+$ cells (Fig. 4B). *Cereberus* and *E-cad*, which are expressed in ES cell-derived endoderm [17], and *Hex*, which is an important transcriptional factor for liver specification [21], were also strongly expressed in the GFP–Bry$^+$/c-kit$^+$ fraction. Taken together, these results suggest that GFP–Bry$^+$/c-kit$^+$ cells cultured in the presence of serum contained the definitive endoderm population.

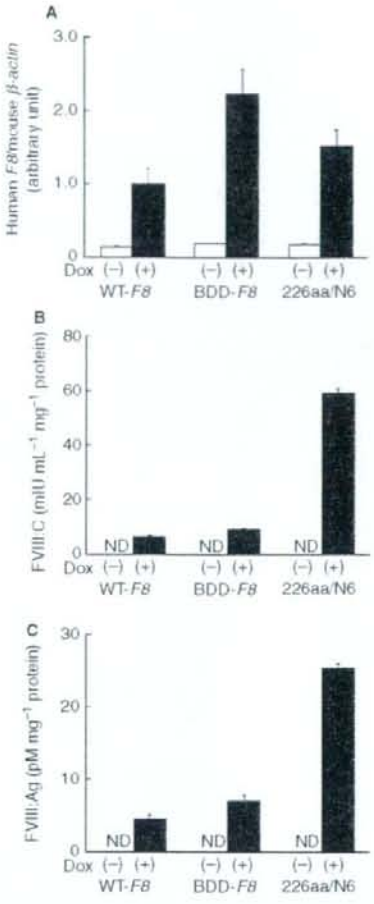

Fig. 3. FVIII secretion from tet-WT-*F8*, tet-BDD-*F8* and tet-226aa/N6 ES cells differentiated under the liver conditions. (A) Real-time PCR analysis of *F8* mRNA levels in tet-WT-*F8*, tet-BDD-*F8* and tet-226aa/N6 ES cells; note that doxycycline (Dox) (1 μg mL^{-1}) induced the highest levels of *F8* mRNA expression in BDD-*F8* cells. (B, C) Secretion of FVIII:C (B) and FVIII:Ag (C) from tet-WT-*F8*, BDD-*F8* and 226aa/N6 ES cells in the presence of 2.5 μg mL^{-1} von Willebrand factor, with or without Dox (1 μg mL^{-1}) induction. The data presented are means of three independent experiments; the error bars represent the SEM. ND, not detected; WT, wild type; BDD, B-domain-deleted.

After sorting, each of the populations derived from tet-226aa/N6 ES cells was reaggregated in SR medium and cultured under the liver conditions. On day 15, EBs derived from GFP–Bry$^+$/c-kit$^+$ cells expressed *Afp* and *Alb* mRNA more strongly than either presorted or GFP–Bry$^+$/c-kit$^-$ cells (Fig. 4C). We then examined the cell populations responsible for the FVIII secretion, and we found that EBs derived from the GFP–Bry$^+$/c-kit$^+$ population were more active for FVIII secretion than the presorted EBs following induction with Dox (Fig. 4D,E). By contrast, GFP–Bry$^-$

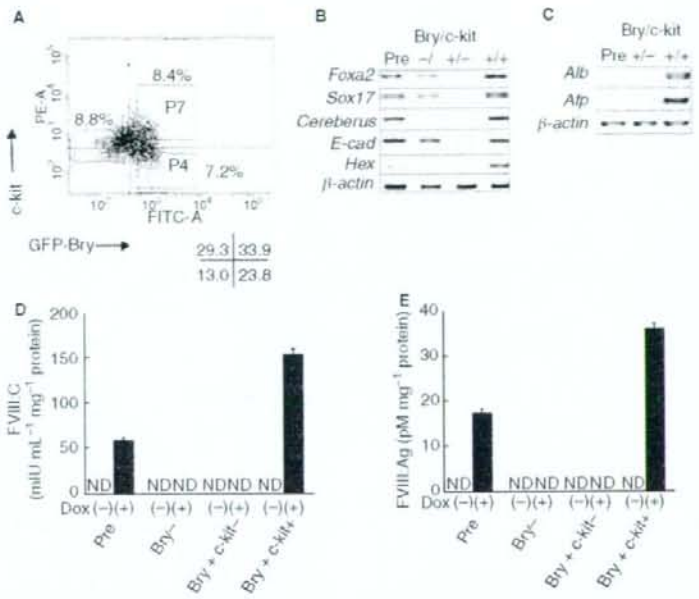

Fig. 4. GFP–Bry$^+$/c-kit$^+$ cells contain the definitive endoderm progenitors and efficiently secrete FVIII. (A) FACS profile for GFP–Bry and c-kit among day 3.5 embryoid bodies cultured in serum-containing medium. (B) Reverse transcription polymerase chain reaction analysis demonstrating the presence of endoderm-related genes in populations derived from presorted cells (pre) or cells sorted on the basis of GFP–Bry and c-kit. (C) Cells from presorted populations or those sorted on the basis of GFP–Bry and c-kit were reaggregated for 1 day, and then cultured in serum replacement medium and replated on day 10. *Alb* and *Afp* mRNA was expressed in GFP–Bry$^+$/c-kit$^+$ cells on day 15. (D, E) Levels of FVIII:C (D) and FVIII:Ag (E) in medium conditioned by presorted cells and those sorted on the basis of GFP–Bry and c-kit, with or without doxycycline (Dox) (1 μg mL^{-1}) induction. The data presented are means of three independent experiments; the error bars represent the SEM. ND, not detected; FITC, fluorescein isothiocyanate; PE, phycoerythrin; GFP, green fluorescent protein; Bry, brachyury.

and GFP–Bry$^+$/c-kit$^-$ cells did not secrete FVIII at all, even after induction with Dox.

Discussion

ES cells capable of secreting human FVIII may represent a unique source for a future cell-based treatment protocol for hemophilia. In the present study, we were able to establish mouse ES cells secreting functional human FVIII with coagulant activity. Tet-WT-*F8* ES cells were established by integrating full-length human *F8* cDNA under the control of the tet operator, which enabled *F8* transcription to be induced by Dox stimulation. We found that levels of FVIII secretion depended on the conditions under which the ES cells were differentiated, regardless of *F8* mRNA expression. Among the conditions that we evaluated, those leading to development of endoderm/liver EBs were the most suitable for efficient FVIII secretion. Furthermore, the efficacy of FVIII secretion was dramatically improved by using 226aa/N6 cDNA, a recently described B-domain variant of *F8* [13]. To our knowledge, this is the first report of an ES/EB system that secretes detectable levels of active human FVIII *in vitro*.

We found it noteworthy that FVIII was present in the supernatant of liver-like EBs, but not in that of undifferentiated ES cells or hematopoietic-like EBs, although the induction of *F8* mRNA was detected in all conditions. It has previously been shown that the transcriptional activity of *F8* is not a critical determinant of plasma FVIII levels, and that mRNA levels are not, themselves, sufficient to predict FVIII secretion [22]. The primary FVIII translation product must be translocated into the lumen of the endoplasmic reticulum (ER), where folding and N-linked glycosylation occurs. Improperly folded FVIII molecules are recognized by chaperones and are not released, but are instead transferred into degradative pathways [23]. Our results indicate that cells with this capacity only appear during differentiation of liver-like EBs, making them more suitable for FVIII secretion than undifferentiated ES cells or hematopoietic-like EBs. Although liver-like EBs expressed hepatocyte-specific marker gene such as *Alb*, *Ttr* and *Tat*, mouse *F8* mRNA was not induced. The reason for this is currently unclear, but liver-like EBs may be still have an immature phenotype for endogeneous *F8* expression. Previous reports have demonstrated that platelets are good targets for the lentivirus-mediated gene therapy of FVIII production [24]. Our hematopoietic EBs were previously showed to contain megakaryocytes, but not platelets [11]. Thus, hematopoietic EBs probably fail to produce FVIII because of the immature differentiation of platelets from megakaryocytes.

We also observed that the 226aa/N6 construct is an extremely useful tool for FVIII production from ES cells. It is known that expression of BDD-*F8* results in a seventeen-fold increase in mRNA levels over WT-*F8*, although it yields only a 1.3-fold increase in the amount of secreted protein [22]. As the reason for imbalance between mRNA and protein levels, BDD-*F8* may have a defect in efficient transfer of the primary translation product from the ER to the Golgi via interaction with the 53-kDa ER–Golgi intermediate compartment protein [25]. To overcome this problem, Miao *et al.* [13] created another bioengineered construct, 226aa/N6. They showed that transfecting COS-1 and CHO cells with 226aa/N6 resulted in a 4–11-fold increase in FVIII secretion, as compared to transfection with BDD-*F8*. Consistent with those studies, we found that BDD-*F8* improved FVIII secretion only about 1.5-fold, as compared to WT-*F8*, whereas tet-226aa/N6 ES cells showed a ten-fold increase in FVIII secretion, as compared to tet-WT-*F8* ES cells. Thus 226aa/N6 appears to provide a significant advantage over BDD-*F8* with respect to FVIII production from ES cells, making it the optimal construct for FVIII secretion.

In our data, the levels of FVIII:C seem to be higher than those of FVIII:Ag, especially in tet-226aa/N6 ES cells. To investigate this discrepancy, we also assessed FVIII:C by a COAtest chromogenic assay (Chromogenix, Mölndal, Sweden) with recombinant FVIII as a standard. In this experiment, FVIII:C was detected at lower levels (about 40–50%) than that evaluated by plasma standard (data not shown). These results were in good accordance with previous reports that FVIII:C level against a plasma standard was higher than that against a recombinant FVIII standard by the chromogenic assay [26]. Thus, the discrepancy between FVIII:C and FVIII:Ag may result from the overestimation of FVIII:C by the APTT clotting assay with plasma standard.

Recently, Gouon-Evans *et al.* [20] demonstrated that the GFP–Bry$^+$/c-kit$^+$ cell population contained definitive endoderm progenitors when ES cells were differentiated in serum-free medium with activin stimulation. Using serum differentiation, we also found that the GFP–Bry$^+$/c-kit$^+$ cell population contained endoderm progenitors and that cells with the liver marker genes *Alb* and *Afp* appeared in this fraction. We further showed that cells differentiated from endoderm progenitor (GFP–Bry$^+$/c-kit$^+$) cells secreted FVIII more efficiently on day 14 of differentiation than presorted cells. By contrast, the sorted GFP–Bry$^-$ (ectoderm progenitor) and GFP–Bry$^+$/c-kit$^-$ (mesoderm progenitor) fractions secreted no FVIII, even after induction with Dox. These findings suggest that cells with the capacity for FVIII production are probably present within endoderm-derived tissue such as liver.

When we consider applying these strategies for human therapy, safety issues will be a big concern. An earlier study showed that grafts containing the undifferentiated ES cells rapidly form teratomas, even when only 0.2% of the cells within the transplanted clusters are positive for the undifferentiated marker SSEA-1 [27]. A recent study succeeded in transplanting ES-derived cardiomyocytes without evidence of teratoma formation in *in vivo* mouse models when selectable markers were employed to eliminate undifferentiated ES cells [28]. Thus, it will probably be necessary to develop a system involving selection markers in our tet-226aa/N6 ES cells for further *in vivo* studies.

In conclusion, we established ES cells secreting human FVIII with tetracycline regulation. The combination of endoderm progenitors, liver condition and 226aa/N6 cDNA could improve production to a significant level of human FVIII from ES cells. Our *in vitro* findings will be the first step for ES cell-based therapy as a potentially useful approach to the treatment of hemophilia A. Further *in vivo* studies are anticipated.

Addendum

S. Kasuda performed laboratory studies, data analysis and interpretation and drafted the manuscript. A. Kubo and Y. Sakurai designed the study, interpreted the data and drafted the manuscript. S. Irion and S. W. Pipe contributed vital new reagents and edited the manuscript. K. Ohashi and K. Tatsumi performed laboratory studies and edited the manuscript. Y. Nakajima, Y. Saito and K. Hatake helped to design the study, interpret the analyses and edit the manuscript. A. Yoshioka and M. Shima contributed critical analytical tools and data interpretation, and edited the manuscript.

Acknowledgements

We thank J. Kato and Y. Bessho for their excellent technical assistance and H. Kinoshita for critically reading the manuscript. We also thank G. Keller in the McEwen Centre for Regenerative Medicine (Toronto, Canada) for critically reading the manuscript and GFP–Bry Ainv18 ES cells. This work was supported in part by Health and Labour Sciences Research Grants for Research on HIV/AIDS from the Ministry of Health, Labour and Welfare (A. Yoshioka), a Bayer Hemophilia Award 2006 (M. Shima), the Mitsubishi Pharma Research Foundation (Y. Sakurai), the Leading Projects from the Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (K. Ohashi) and the Uehara Memorial Foundation (A. Kubo).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Antonarakis SE. Molecular genetics of coagulation factor VIII gene and haemophilia A. *Haemophilia* 1998; **4**(Suppl. 2): 1–11.
2 Manco-Johnson M. Hemophilia management: optimizing treatment based on patient needs. *Curr Opin Pediatr* 2005; **17**: 3–6.
3 Lillicrap D, Vandendriessche T, High K. Cellular and genetic therapies for haemophilia. *Haemophilia* 2006; **12**(Suppl. 3): 36–41.
4 Sarkar R, Tetreault R, Gao G, Wang L, Bell P, Chandler R, Wilson JM, Kazazian HH Jr. Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood* 2004; **103**: 1253–60.

5 Kaiser J. Gene therapy. Side effects sideline hemophilia trial. *Science* 2004; **304**: 1423–5.

6 Schuettrumpf J, Liu JH, Couto LB, Addya K, Leonard DG, Zhen Z, Sommer J, Arruda VR. Inadvertent germline transmission of AAV2 vector: findings in a rabbit model correlate with those in a human clinical trial. *Mol Ther* 2006; **13**: 1064–73.

7 Ko S, Tanaka I, Kanehiro H, Kanokogi H, Ori J, Shima M, Yoshioka A, Giles A, Nakajima Y. Preclinical experiment of auxiliary partial orthotopic liver transplantation as a curative treatment for hemophilia. *Liver Transpl* 2005; **11**: 579–84.

8 Ohashi K, Waugh JM, Dake MD, Yokoyama T, Kuge H, Nakajima Y, Yamanouchi M, Naka H, Yoshioka A, Kay MA. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. *Hepatology* 2005; **41**: 132–40.

9 Kumaran V, Benten D, Follenzi A, Joseph B, Sarkar R, Gupta S. Transplantation of endothelial cells corrects the phenotype in hemophilia A mice. *J Thromb Haemost* 2005; **3**: 2022–31.

10 Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 2002; **109**: 29–37.

11 Kubo A, Chen V, Kennedy M, Zahradka E, Daley GQ, Keller G. The homeobox gene HEX regulates proliferation and differentiation of hemangioblasts and endothelial cells during ES cell differentiation. *Blood* 2005; **105**: 4590–7.

12 Shibata M, Shima M, Morichika S, McVey J, Tuddenham EG, Tanaka I, Suzuki H, Nogami K, Minamoto Y, Hato T, Saenko EL, Scandella D, Yoshioka A. An alloantibody recognizing the FVIII A1 domain in a patient with CRM reduced haemophilia A due to deletion of a large portion of the A1 domain DNA sequence. *Thromb Haemost* 2000; **84**: 442–8.

13 Miao HZ, Sirachainan N, Palmer L, Kucab P, Cunningham MA, Kaufman RJ, Pipe SW. Bioengineering of coagulation factor VIII for improved secretion. *Blood* 2004; **103**: 3412–19.

14 Fehling HJ, Lacaud G, Kubo A, Kennedy M, Robertson S, Keller G, Kouskoff V. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development* 2003; **130**: 4217–27.

15 Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. Development of definitive endoderm from embryonic stem cells in culture. *Development* 2004; **131**: 1651–62.

16 Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *J Biol Chem* 1988; **263**: 6352–62.

17 Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M, Nakao K, Chiba T, Nishikawa S. Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development* 2005; **132**: 4363–74.

18 Pittman DD, Alderman EM, Tomkinson KN, Wang JH, Giles AR, Kaufman RJ. Biochemical, immunological, and in vivo functional characterization of B-domain-deleted factor VIII. *Blood* 1993; **81**: 2925–35.

19 Toole JJ, Pittman DD, Orr EC, Murtha P, Wasley LC, Kaufman RJ. A large region (approximately equal to 95 kDa) of human factor VIII is dispensable for in vitro procoagulant activity. *Proc Natl Acad Sci USA* 1986; **83**: 5939–42.

20 Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, Shafritz DA, Keller G. BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. *Nat Biotechnol* 2006; **24**: 1402–11.

21 Barbera JPM, Clements M, Thomas P, Rodriguez T, Meloy D, Kioussis D, Beddington RSP. The homeobox gene Hex is required in definitive endoderm tissues for normal forebrain, liver and thyroid formation. *Development* 2000; **127**: 2433–45.

22 Pittman DD, Marquette KA, Kaufman RJ. Role of the B domain for factor VIII and factor V expression and function. *Blood* 1994; **84**: 4214–25.

23 Pipe SW, Morris JA, Shah J, Kaufman RJ. Differential interaction of coagulation factor VIII and factor V with protein chaperones calnexin and calreticulin. *J Biol Chem* 1998; **273**: 8537–44.

24 Shi Q, Wilcox DA, Fahs SA, Fang J, Johnson BD, Du LM, Desai D, Montgomery RR. Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. *J Thromb Haemost* 2007; **5**: 352–61.

25 Pipe SW, Saint-Remy JM, Walsh CE. New high-technology products for the treatment of haemophilia. *Haemophilia* 2004; **10**(Suppl. 4): 55–63.

26 Hubbard AR, Bevan SA, Weller LJ. Potency estimation of recombinant factor VIII: effect of assay method and standard. *Br J Haematol* 2001; **113**: 533–6.

27 Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol* 2005; **166**: 1781–91.

28 Kolossov E, Bostani T, Roell W, Breitbach M, Pillekamp F, Nygren JM, Sasse P, Rubenchik O, Fries JW, Wenzel D, Geisen C, Xia Y, Lu Z, Duan Y, Kettenhofen R, Jovinge S, Bloch W, Bohlen H, Welz A, Hescheler J, *et al.* Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium. *J Exp Med* 2006; **203**: 2315–27.

Successful *in vivo* propagation of factor IX-producing hepatocytes in mice: Potential for cell-based therapy in haemophilia B

Kohei Tatsumi[1], Kazuo Ohashi[2, 5], Miho Kataoka[3], Chise Tateno[3, #], Masaru Shibata[1], Hiroyuki Naka[1], Midori Shima[1], Michiyoshi Hisanaga[2], Hiromichi Kanehiro[2], Teruo Okano[5], Katsutoshi Yoshizato[3, 4, #], Yoshiyuki Nakajima[2], Akira Yoshioka[1]

[1]Department of Pediatrics, Nara Medical University, Nara, Japan; [2]Department of Surgery, Nara Medical University, Nara, Japan; [3]Yoshizato Project, Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER), Hiroshima Prefectural Institute of Industrial Science and Technology, Hiroshima, Japan; [4]Graduate School of Science, Hiroshima University, Hiroshima, Japan; [5]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan

Summary

Cell-based therapies using isolated hepatocytes have been proposed to be an attractive application in the treatment of haemophilia B due to the normal production of coagulation factor IX (FIX) in these particular cells. Current cell culture technologies have largely failed to provide adequate isolated hepatocytes, so the present studies were designed to examine a new approach to efficiently proliferate hepatocytes that can retain normal biological function, including the ability to synthesize coagulation factors like FIX. Canine or human primary hepatocytes were transplanted into urokinase-type plasminogen activator-severe combined immunodeficiency (uPA/SCID) transgenic mice. Both donor hepatocytes from canines and humans were found to progressively proliferate in the recipient mouse livers as evidenced by a sharp increase in the circulating blood levels of species-specific albumin, which was correlated with the production and release of canine and human FIX antigen levels into the plasma. Histological examination confirmed that the transplanted canine and human hepatocytes were able to proliferate and occupy >80% of the host livers. In addition, the transplanted hepatocytes demonstrated strong cytoplasmic staining for human FIX, and the secreted coagulation factor IX was found to be haemostatically competent using specific procoagulant assays. In all, the results from the present study indicated that developments based on this technology could provide sufficient FIX-producing hepatocytes for cell-based therapy for haemophilia B.

Keywords

Haemophilia A/B, haemophilia therapy, coagulation factors, hepatology

Thromb Haemost 2008; 99: 883–891

Introduction

Haemophilia B is a rare X-chromosome-linked recessive bleeding disorder, caused by a failure in the production of functional coagulation factor IX (FIX), and this disease affects ~1 in 30,000 males (1, 2). The main clinical manifestation of this disease is similar to haemophilia A (factor VIII deficiency), and under severe conditions the affected patient can be found to have unpredictable, recurrent, spontaneous bleeding in various areas, including soft tissues, major joints and occasionally in internal organs. In these circumstances, the onset and progression of chronic haemoarthropathy leads to a marked disruption in the physical and social aspects of the affected patients. Standard treatment for haemophilia B is either on-demand or prophylactic therapy with plasma-derived or recombinant human FIX concentrates. This type of treatment requires frequent intravenous infusion, which can be a potential biohazard from blood-borne viral infections to the patient if the infusate is derived from a heterogeneous population of human blood. In addition, the high cost of commercial concentrates and the life-long requirement for replacement therapy can have a significant impact on economic resources. In an attempt to resolve these difficulties, longer acting

Correspondence to:
Kazuo Ohashi, MD, PhD
Institute of Advanced Biomedical Engineering and Science
Tokyo Women's Medical University
8–1 Kawada-cho, Shinjuku-ku, Tokyo, 162–8666, Japan
Tel.: +81 3 3353 8111, ext 66214, Fax: +81 3 3359 6046
E-mail: ohashi@abmes.twmu.ac.jp

Present address: PhoenixBio Co. Ltd, Hiroshima, Japan.

Financial support:
This study was supported in part by The Leading Projects (K.O. and T.O.) and Grant-in-Aid (no. 18591957 to K.O.) from the Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan, grants for AIDS Research from the Ministry of Health, Labor and Welfare of Japan (A.Y.), and grant for CLUSTER (K.Y.).

Received September 12, 2007
Accepted after major revision March 12, 2008

Prepublished online April 9, 2008
doi:10.1160/TH07-09-0559

and safer therapeutic strategies have been investigated. For example, gene therapy using viral vectors has been extensively studied in the past decade (3), and although therapeutic and long-term efficacy has been demonstrated in animal models (4–12), clinical trials have not conclusively shown long-term therapeutic benefit (13, 14). It seems likely, therefore, that alternate therapeutic options will need to be developed.

Recent clinical success with liver transplantation in haemophilia has encouraged further investigation into cell-based therapies (15–17). In haemophilia B patients, elevations in biologically active FIX levels from <1.0% to >1.0%, can alter the phenotype from severe to moderate resulting in a marked improvement in the symptomology and quality of life (1). Coagulation FIX is synthesized in hepatocytes (18), and so cell-based therapies using isolated hepatocytes could provide therapeutic potential. Hepatocytes also produce other coagulation factors, such as factors VII and VIII (19–24), and it may be that this type of treatment could have broader applications to not only haemophlia B, but other coagulation deficiencies. Recently, we have adopted several approaches to bioengineer functional liver tissue *in vivo* (25–30). We have demonstrated that isolated hepatocytes transplanted under the kidney capsule in haemophilia A mice produced therapeutic plasma FVIII activity and corrected the phenotypic defect (28). Dhawan et al. (31) also recently described the therapeutic benefits of hepatocyte transplantation in congenital factor VII deficiency, and the relative technical simplicity of cell-based therapy may offer a significant and technological advantage.

One of the major hurdles in establishing this type of therapy is the limited availability of biologically functional hepatocytes. At present, the number of donor livers remains severely restricted and even if they are available, these livers are frequently of marginal quality (32). Current procedures for the culture of primary hepatocytes do not appear to support extensive cell proliferation (33), so methods to circumvent this problem have recently been studied, but their role to treat haemophilia were not examined. Isolated hepatocytes were genetically modified via transfection with an immortalizing gene, such as simian virus 40 large T antigen, to promote long-term survival (34), but FIX gene expression and production was not investigated. Although the genetic manipulation of hepatocytes can be achieved following isolation *in vitro*, this type of approach to promote hepatocyte proliferation is not a trivial matter *in vivo*. Towards this end, methods to provide proliferative stimuli has been studied *in vivo*, such as a reduction in existing liver mass, or alternatively in a condition where there is likely to be a selective advantage for transplanted cells to proliferate (26, 28). Due to these limitations, we investigated a different method to isolate and proliferate hepatocytes that can retain the hepatic machinery to sustain the synthesis of coagulation factors, such as FIX. In the present study, we studied whether transplantation of canine or human primary hepatocytes into urokinase-type plasminogen activator-severe combined immunodeficiency (uPA/SCID) transgenic mice could enhance the production of coagulation factor IX. The uPA/SCID mouse has been previously shown to have hepatic parenchymal cell damage, which results in the continuous release of regenerative stimuli (35), so we believed that the hepatic environment may be more conducive to the engraftment of *in vitro* isolated hepatocytes. The functionality of the transplanted hepatocytes was assessed in terms of FIX mRNA and protein production and biological activity as a means to treat haemophilia B.

Materials and methods

Animals

Normal beagles were purchased from Oriental BioService, Inc. (Kyoto, Japan). C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). uPA/SCID mice were generated at Hiroshima Prefectural Institute of Industrial Science and Technology (Higashihiroshima, Hiroshima, Japan) as described previously (35). Genotyping for the presence of the uPA transgene in the SCID mice was confirmed by polymerase chain reaction (PCR) assay of isolated genomic DNA as described previously (35, 36). Experimental protocols were developed in accordance with the guidelines of the local animal committees located at both Hiroshima Prefectural Institute of Industrial Science and Technology and Nara Medical University.

Hepatocyte isolation

Canine hepatocytes were isolated from livers (~100 g piece) harvested from two normal beagles (Dog 1: 7-year-old male and Dog 2: 1-year-old female) by a two-step perfusion method using 0.05% collagenase (Collagenase S1, Nitta Gelatin, Osaka, Japan) as described previously (25, 27). Cells were then filtered and hepatocytes were separated from non-parenchymal cells by sequential low speed centrifugation at 50 x g followed by Percoll (Percoll™, Amersham Biosciences, Uppsala, Sweden) isodensity centrifugation. The viabilities of the isolated canine hepatocytes were 96.5% and 98.0% as determined by the trypan blue exclusion test. Hepatocytes were kept at 4°C until transplantation. Human hepatocytes, isolated from a one-year-old white male and a six-year-old Afro-American female, were purchased from In Vitro Technologies (Baltimore, MD, USA). The cryopreserved hepatocytes were thawed and suspended in transplant medium (35, 37). The viabilities of thawed human hepatocytes were determined to be 64.4% and 49.2%, respectively.

Transplantation of hepatocytes for the creation of canine- or human-chimeric mice

One day prior to transplantation and one week after transplantation, the uPA/SCID mice, 20 to 30 days old, received intraperitoneal injections of 0.1 mg of anti-asialo GM1 rabbit serum (Wako Pure Chemical Industries Ltd., Osaka, Japan) to inhibit recipient natural killer cell activity against the transplanted hepatocytes. Viable canine- (1.0×10^6) or human- (0.75×10^6) hepatocytes were transplanted using an infusion technique into the inferior splenic pole in which the transplanted cells flow from the spleen into the liver via the portal system. After transplantation, the uPA/SCID mice were treated with nafamostat mesilate to inhibit complement factors activated by canine or human hepatocytes as previously described (35).

Measurement of plasma levels of albumin, FIX antigen and FIX activity

Periodically, retroorbital bleeding was performed in recipient mice, and the blood was collected in a tube containing 0.1 vol

3.8% sodium citrate. Plasma samples were stored at −80°C until analyzed. To assess the proliferating status of transplanted canine hepatocytes, we determined the plasma levels of canine albumin in the recipient plasma by ELISA using primary goat anti-dog albumin and secondary HRP-conjugated goat anti-dog albumin antibodies (Bethyl Laboratories Inc., Montgomery, TX, USA), respectively. For the assessment of proliferation in transplanted human hepatocytes, we similarly measured the blood levels of human albumin by ELISA (Human Albumin ELISA Quantitation kit, Bethyl Laboratories Inc.). The proportion of proliferating donor hepatocytes in the recipient liver (repopulation rate) was determined based on blood albumin levels (35, 38). Human and canine FIX antigen (FIX:Ag) were measured in recipient plasma by ELISA (Asserachrom IX:Ag, Diagnostica Stago, Asnières, France). Human FIX:Ag levels were measured according to the instructions provided by the manufacturer, and canine FIX:Ag levels were quantified by elongating the enzymatic color reaction step. No cross-reactivity with pooled mouse plasma was observed in this ELISA. FIX activity (FIX:C) was measured by one-stage clotting assay based on the activated partial thromboplastin time using human FIX-deficient plasma (bioMerieux Inc., Durham, NC, USA). Pooled canine plasma collected from 75 normal dogs, and normal human plasma (Verify 1, bioMerieux Inc.) were used as reference standards.

Immunohistochemistry for albumin and FIX

Formalin-fixed, paraffin-embedded liver sections from mice transplanted with canine hepatocytes were sectioned and incubated with a primary goat antibody against canine albumin (Bethyl Laboratories Inc.) at a dilution of 1:1,000. The bound antibody was detected by the avidin-biotin complex immunoperoxidase technique using an ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) followed by developing with DAB (3, 3'-diaminobenzine tetrahydrochloride). Expression of human FIX in recipient mice was determined by immunofluorescent staining of frozen liver sections embedded in O.C.T compound

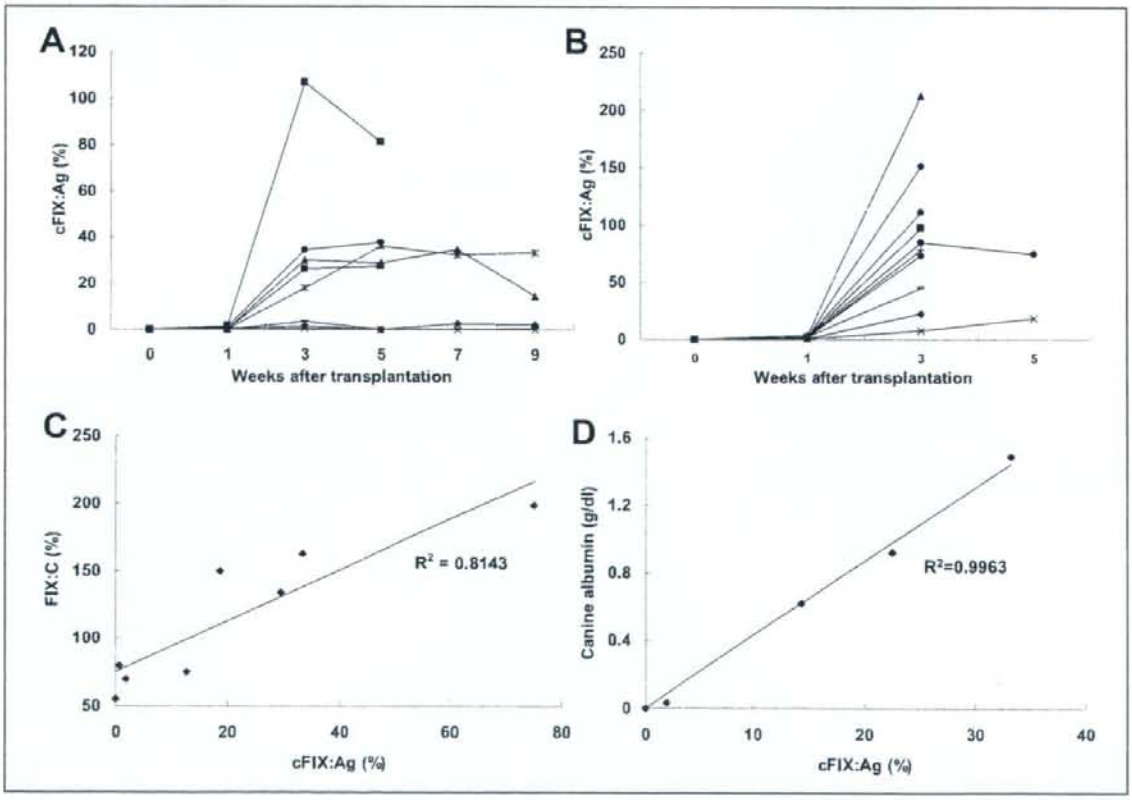

Figure 1: Proliferation of transplanted canine hepatocytes in uPA/SCID mouse livers assessed by recipient plasma analyses. A, B) Plasma canine factor IX (FIX) antigen (cFIX:Ag) levels in uPA/SCID mice after transplantation of hepatocytes isolated from a seven-year-old dog (A) and a one-year-old dog (B) (n=8, 10 in A and B, respectively) (% of pooled normal canine plasma). C) Relationship between total plasma FIX coagulation activity (FIX:C; reflecting both murine and canine FIX activities) (% of normal human plasma) and plasma cFIX:Ag levels of uPA/SCID mice transplanted with canine hepatocytes. D) Relationship between plasma canine albumin concentrations and plasma cFIX:Ag levels of uPA/SCID mice transplanted with canine hepatocytes.

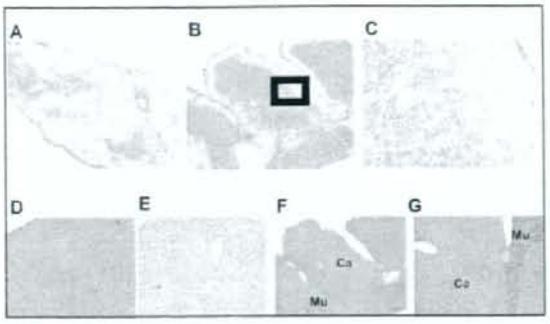

Figure 2: Mouse liver chimerism with proliferated canine hepatocytes. A-E) Immunohistochemical staining of canine albumin in liver sections of uPA/SCID mice transplanted with canine hepatocytes. Representative photomicrographs from a recipient mouse with low plasma cFIX:Ag (2.0% of normal canine plasma) (A) and a mouse with high plasma cFIX:Ag (33.2% of normal canine plasma) (B). C) Higher magnification view of the area outlined in (B). Canine albumin staining of positive control (normal dog liver) (D) and negative control (non-transplanted uPA/SCID mouse liver) (E) indicate the antibody used is specific for canine albumin. F, G) Hematoxylin and Eosin staining on the serial sections of mouse liver from (B). Ca, transplanted canine hepatocytes; Mu, recipient murine liver tissue. Original magnifications, ×40 (A, B, F), ×100 (D,E), and ×200 (C, G).

(Sakura Finetek, Torrance, CA, USA). The sections were incubated overnight at 4°C with the goat anti-human FIX antibody (Affinity Biologicals, Hamilton, ON, Canada) followed by Alexa Fluor 555 rabbit anti-goat IgG (Molecular Probes, Carlsbad, CA, USA) for 60 minutes. Stained sections were subsequently imaged using an Olympus BX51 microscope (Tokyo, Japan) and photographed using an Olympus DP70 digital camera with DP controller and DP manager computer software.

Quantitative real-time PCR

Total RNA was extracted from the liver of all recipient mice, and normal human and canine liver samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Normal human liver tissue portions were obtained from surgical specimens at liver surgery for metastatic liver tumours after acquiring written informed consent for the experimental use of harvested liver samples. Extracted RNA (1 μg) was reverse transcribed using oligo $d(T)_{16}$ primers and Omniscript RT Kit (Qiagen). First-strand cDNA samples were subsequently subjected to PCR amplification using the PRISM 7700 Sequence Detector (Applied Biosystems Japan Ltd., Tokyo, Japan). Canine glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and canine FIX sequences were detected using the following primers. The PCR primers for canine GAPDH sequence were forward, 5'CCCCACCCCCAATGTATCA3', reverse, 5'GTCGTCATATTTGGCAGCTTTCT3', and probe, 5'TGTGGATCTGACCTGCCGCCTG3'.
The primers for canine FIX sequence were forward, 5'GTTGTTGGTGGAAAAGATGCC3', reverse, 5'TGCATCAACTTTCCCATTCAAA3', probe, 'CCAGGTCAATTCCCTTGGCAGGTCC3'. TaqMan probes and primers for human sequences were Hs99999905_m1 (GAPDH) and Hs00609168_m1 (FIX) (TaqMan Gene Expression Assay, Applied Biosystems). The relative RNA copy numbers of canine FIX and human FIX in each transplanted mouse were calculated in terms of canine FIX / canine GAPDH or human FIX / human GAPDH expression ratio, respectively. RNA expression of murine FIX and murine GAPDH, combined with cDNA synthesis and real-time PCR using TaqMan probes, Mm99999915_g1 (murine GAPDH) and Mm01308427_m1 (murine FIX) (Applied Biosystems), were similarly assessed in hepatectomy experiments (see below).

Hepatectomy experiment

For the purpose of investigating the FIX mRNA expression during liver regeneration, liver proliferation stimuli was induced by performing a 70% partial hepatectomy on C57BL6 wild-type mice (n=6) as described previously (39). The resected liver lobes were used as our control for a liver sample under quiescence while the remnant liver lobes removed two days after hepatectomy were used as our proliferating samples. Mouse FIX mRNA and mouse GAPDH mRNA expression was assessed on both quiescent and proliferating liver samples as described above.

Statistical analysis

Significant differences were tested by the Wilcoxon t-test between paired groups and by the Mann-Whitney U-test between unpaired groups. Differences between three or more groups were tested by the Kruskal Wallis H-test. If the probability (p) value was less than 0.05, the Mann-Whitney U-test with Bonferroni correction was used to compare each individual group with the appropriate control. All statistical analyses were performed using Excel (Microsoft) with ystat2006 software (Igakutosyo-syuppan, Tokyo, Japan). $P<0.05$ was considered significant.

Results

Proliferation of FIX-producing canine hepatocytes in uPA/SCID mouse livers

Canine hepatocytes isolated from a seven-year-old and a one-year-old beagle were transplanted into uPA/SCID mice (n=8 and 10, respectively). Canine FIX:Ag was detected in the plasma of five out of eight mice three weeks after transplantation with the isolated hepatocytes from the seven-year-old beagle. In four out of the five mice, the FIX:Ag levels reached between 20–40% of normal canine plasma levels for FIX:Ag (Fig. 1A). One transplanted mouse was detected to have nearly 100% of normal canine plasma FIX:Ag levels. In general, the uPA/SCID mice that received hepatocytes from the one-year-old beagle demonstrated a greater rise in the circulating canine FIX:Ag, and 70% of the mice (7 out of 10) showed levels greater than 50% of normal levels three weeks after transplantation (median: 81.8%; Fig. 1B).

Plasma FIX:C was measured using a one-stage clotting assay. The FIX:C of normal canine pooled plasma and untreated uPA/SCID mouse plasma (n=4) was approximately 200% and 50% of normal human plasma, respectively. The FIX:C in the recipient uPA/SCID mice with high canine FIX:Ag levels was greater than in untreated mice or recipient uPA/SCID mice with low FIX:Ag levels ($R^2=0.8143$) (Fig. 1C). These observations confirmed that the secreted FIX protein had functional coagulation activity.