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ORIGINAL ARTICLE

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

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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-kit$^+$ cells during EB differentiation with serum contain an endoderm progenitor population. When GFP–Bry$^+$/c-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.

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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).

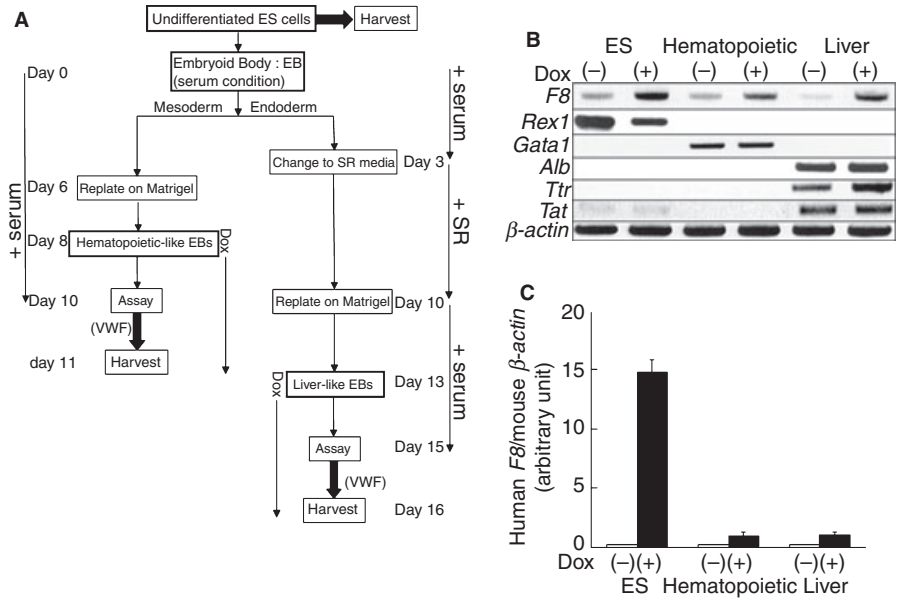

Fig. 1. Expressions of WT-*F8* mRNA by doxycycline (Dox) stimulation in undifferentiated embryonic stem (ES) cells, hematopoietic-like embryoid bodies (EBs) and liver-like EBs. (A) Schema of the experimental protocol. (B) Reverse transcription polymerase chain reaction (RT-PCR) analysis of variable marker 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

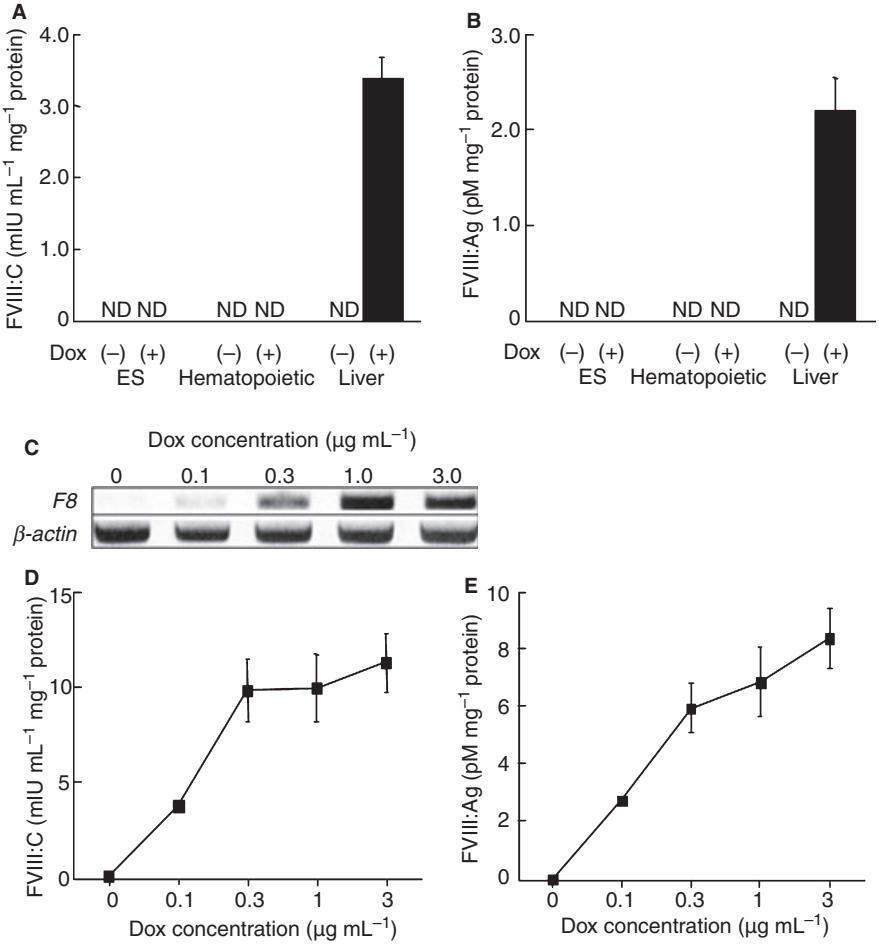

Fig. 2. FVIII:C and FVIII:Ag levels in undifferentiated embryonic stem (ES) cells, hematopoietic-like embryoid bodies (EBs) and liver-like EBs with or without doxycycline (Dox) stimulation. (A, B) FVIII:C (A) and FVIII:Ag (B) levels in media conditioned by cells cultured under the three differentiation 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.

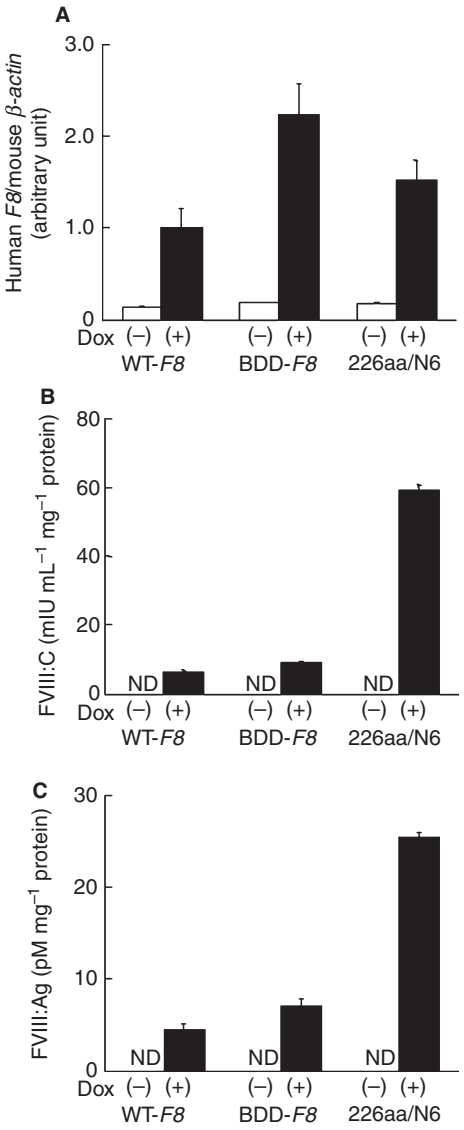

Fig. 3. FVIII secretion from tet-WT-*F8*, tet-BDD-*F8* and tet-226aa/N6 ES cells differentiated under the liver conditions. (A) Real-time PCR analysis of *F8* mRNA levels in tet-WT-*F8*, tet-BDD-*F8* and tet-226aa/N6 ES cells; note that doxycycline (Dox) (1 μg mL^{-1}) induced the highest levels of *F8* 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$^-$

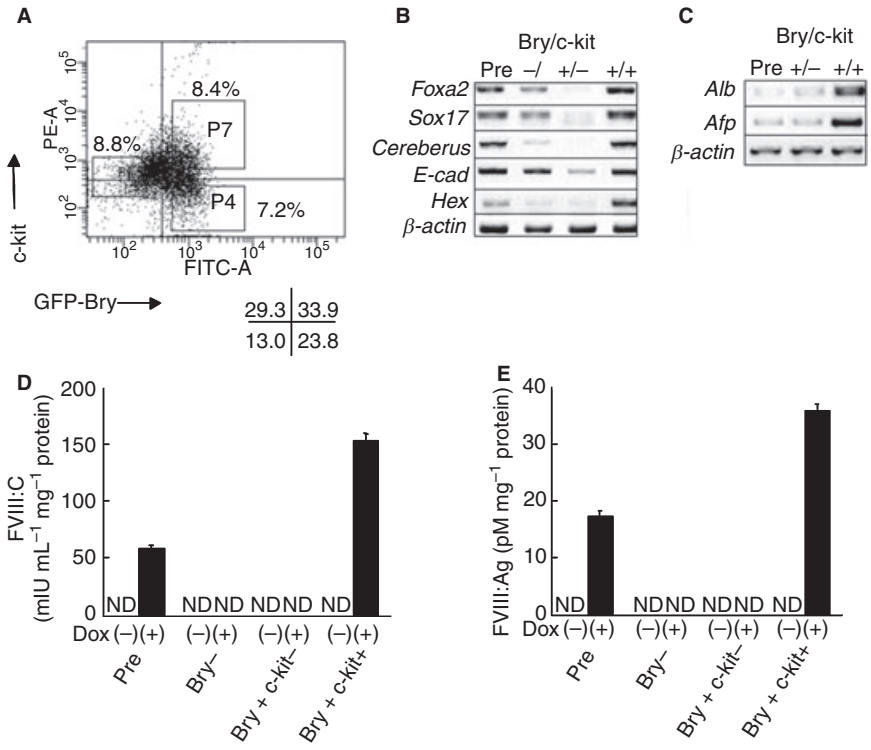

Fig. 4. GFP–Bry$^+$/c-kit$^+$ cells contain the definitive endoderm progenitors and efficiently secrete FVIII. (A) FACS profile for GFP–Bry and c-kit among day 3.5 embryoid bodies cultured in serum-containing medium. (B) Reverse transcription polymerase chain reaction analysis demonstrating the presence 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.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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