Extrahepatic sources of factor VIII potentially contribute to the coagulation cascade correcting the bleeding phenotype of mice with hemophilia A

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ABSTRACT

A large fraction of factor VIII in blood originates from liver sinusoidal endothelial cells although extrahepatic sources also contribute to plasma factor VIII levels. Identification of cell-types other than endothelial cells with the capacity to synthesize and release factor VIII will be helpful for therapeutic approaches in hemophilia A. Recent cell therapy and bone marrow transplantation studies indicated that Küpffer cells, monocytes and mesenchymal stromal cells could synthesize factor VIII in sufficient amount to ameliorate the bleeding phenotype in hemophilic mice. To further establish the role of blood cells in expressing factor VIII, we studied various types of mouse and human hematopoietic cells. We identified factor VIII in cells isolated from peripheral and cord blood, as well as bone marrow. Co-staining for cell type-specific markers verified that factor VIII was expressed in monocytes, macrophages and megakaryocytes. We additionally verified that factor VIII was expressed in liver sinusoidal endothelial cells and endothelial cells elsewhere, e.g., in the spleen, lungs and kidneys. Factor VIII was well expressed in sinusoidal endothelial cells and Küpffer cells isolated from human liver, whereas by comparison isolated human hepatocytes expressed factor VIII at very low levels. After transplantation of $CD34^+$ human cord blood cells into NOD/SCIDγNull-hemophilia A mice, fluorescence activated cell sorting of peripheral blood showed >40% donor cells engrafted in the majority of mice. In these animals, plasma factor VIII activity 12 weeks after cell transplantation was up to 5% and nine of 12 mice survived after a tail clip-assay. In conclusion, hematopoietic cells, in addition to endothelial cells, express and secrete factor VIII: this information should offer further opportunities for understanding mechanisms of factor VIII synthesis and replenishment.

Introduction

The X-linked bleeding disorder of hemophilia A (HA) is characterized by coagulation factor VIII (FVIII) deficiency.[1] Currently, HA is treated by administration of plasma-derived or recombinant FVIII,[2] but this strategy is complicated by the development of inhibitory antibodies in 30-40% of patients affected by the severe form of the disease.[3] Curative gene and cell therapies are, therefore, of interest for HA. It would be useful for such therapies to delineate the cell types capable of producing FVIII in necessary amounts.[4] This study was aimed to determine whether hematopoietic lineage cells could serve roles in the production of FVIII. For several decades, liver was considered the primary site of FVIII production since orthotopic liver transplantation corrected HA.[5] On the other hand, transplantation of liver from hemophilic donors, either dogs[6] or humans,[7] into healthy subjects does not cause hemophilia, indicating that FVIII is also produced in extrahepatic sites. Recent studies using a cell therapy approach[8,9] or cell type-specific knockout experiments indicated that FVIII is produced largely in liver sinusoidal endothelial cells (LSEC);[10,11] although FVIII mRNA was present in endothelial cells of kidneys, spleen and lungs, it was absent in endothelial cells of the brain and heart.[10,12-15] These findings were in agreement with studies showing that hemophilic patients benefited from transplantation of the spleen in the long-term.[16,17] On the other hand, early studies in hemophilic dogs did not show long-term correction and other reports described the spleen as only a store for FVIII-expressing cells.[18,19] For instance, the spleen was found to harbor large numbers of monocytes/macrophages but the physiological significance of FVIII expression in macrophages[20] or peripheral blood mononuclear cells[21] is unclear. Nonetheless, is it noteworthy that FVIII was originally cloned with RNA from a T-cell line.[22] Recently, bone marrow (BM) transplantation was demonstrated to correct the bleeding phenotype in HA mice, in part through donor-derived monocytes/macrophages and mesenchymal stromal cells.[23,24] Further investigations into the role of hematopoietic cells in FVIII expression are, therefore, appropriate.

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Although liver-directed gene therapy for hemophilia captured interest, expressing FVIII in other cell types, such as hematopoietic stem cells[25,26] and platelets,[27-30] is also considered to be relevant. In several mouse studies, expression of human FVIII in hematopoietic stem/progenitors cells corrected hemophilia A.[25,31-33] The advantages of expressing FVIII in platelets are these cells' involvement in early hemostasis and the fact that they serve as a major site for storage of FVIII.[34] In megakaryocytes and endothelial cells the presence of von Willebrand factor should be helpful for stabilizing FVIII. It is possible that FVIII in platelets may not cause the development of neutralizing antibodies.[35] However, whether megakaryocytes may natively express FVIII has not yet been established.

Here, we focused particularly on what cells of the hematopoietic lineage may produce and release FVIII. This was investigated by differentiating monocytes from human or mouse blood into macrophages *in vitro*, and generating myeloid cells from BM or cord blood stem/progenitor cells. The significance of FVIII expression in these cell types was examined by cell transplantation assays for their ability to replace FVIII in severely immunocompromised severely immunocompromised HA mice.

Methods

Polyclonal antibodies

The production and analysis of antibodies is described in the *Online Supplementary Methods*.

Human cell isolation and differentiation

Details on the procedures for isolation and culture of liver cells are given in the *Online Supplementary Methods*. Peripheral blood mononuclear cells were isolated with Ficoll-Paque™ Premium (GE Healthcare). To promote adhesion of isolated monocytes, peripheral blood mononuclear cells were cultured in serum-free RPMI medium. After 30 min, the medium was changed with RPMI containing 5% fetal bovine serum, and cells were cultured for 12 h. To generate macrophages, cells were cultured in DMEM with 10% fetal bovine serum, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 0.25 mM HEPES (Lonza), and 10 ng/mL macrophage colony-stimulating factor.

Hematopoietic stem cells were isolated from mononuclear cord blood cells using MACS® CD34 MicroBeads Kit (Miltenyi). Procedures to obtain macrophages and megakaryocytes from $CD34^+$ cells are described in the *Online Supplementary Methods.*

For xenotransplantation studies, $CD34^+$ cells were cultured for 24 h in STEM-SPAM containing 50 ng/mL human thrombopoietin, 50 ng/mL human stem cell factor, 50 ng/mL human interleukin-3 and 50 ng/mL human Flt3-ligand.

Mouse cell isolation and differentiation

Total peripheral blood mononuclear cells were plated at a density of $2x10^6$ cells/mL in DMEM containing 10% fetal bovine serum. BM cells were differentiated into macrophages (BM-DM) in IMDM containing 10% fetal bovine serum and 5 ng/mL recombinant murine macrophage colony-stimulating factor. For studies, BM-DM were analyzed 5-7 days later.

For megakaryocyte differentiation, c-Kit^+ cells were isolated using mouse CD117-MicroBeads kit (Miltenyi) from total BM cells. Isolated cells were cultured for 2 days in STEM-SPAM containing 20 ng/mL stem cell factor. Then 10^6 cells/mL were cultured for 3-4 days in STEM-SPAM containing 100 ng/mL thrombopoietin, 10 ng/mL interleukin-6 and 10 ng/mL interleukin-11.

RNA isolation, reverse transcriptase polymerase chain reaction and quantitative polymerase chain reaction

RNA was isolated by Isol-RNA Lysis Reagent (5PRIME). One microgram of RNA was reverse-transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative polymerase chain reaction (PCR) was performed with GoTaq® qPCR Master Mix (Promega). Primers and amplification protocols are described in the *Online Supplementary Methods*.

Immunostaining

Mouse tissues were fixed in 4% paraformaldehyde for 2 h at 4°C, equilibrated in sucrose, and embedded in cryostat embedding medium (Bio-Optica). Human organs were paraffin-embedded. Cells were plated on 12 mm glass coverslips. Megakaryocytes were cytospun onto glass slides. Procedures for immunohistochemistry/immunofluorescence and antibodies are described in the *Online Supplementary Methods*.

Cell transplantation studies in mice

Animal studies were approved by the Animal Care and Use Committee of the Università del Piemonte Orientale "A.Avogadro" (Novara, Italy). For xenotransplantation, we used immunocompromised NOD/SCID HA (NSHA) mice transferred from Einstein College in New York to the Università del Piemonte Orientale.[8] NOD/SCID-γNull HA mice (NSG-HA) were generated by crossing these immunocompromised NOD/SCID HA mice with NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}*/SzJ* (γNull) mice from Jackson Laboratories (Bar Harbor, Maine, USA) since this background is superior for transplanting human cells.[36] $CD11b^+$ human cord blood-derived mononuclear cells ($15x10^6$) were injected into the tail vein of 6- to 8-week old NSG-HA mice. For human $CD34^+$ transplantation studies, 10- to 12-week old NSG-HA mice were conditioned with 50 mg/kg busulfan and 24 h later $3\text{-}6x10^5$ $CD34^+$ cells per mouse were injected intravenously.

Factor VIII activity

To evaluate FVIII activity, the activated partial thromboplastin time (aPTT) was measured in plasma samples and a chromogenic assay was performed using a Coatest® SP4 FVIII-kit (Chromogenix). Standard curves were generated by serial dilution of commercial human FVIII (Kogenate®, Bayer) for the chromogenic assay and by serial dilution of pooled human plasma mixed with HA mouse plasma for the aPTT assay. Results are expressed as international units (IU) for the chromogenic assay and as percentage of normal FVIII activity for the aPTT assay.

Results

Factor VIII expression in bone marrow and peripheral blood cells

We first investigated whether human BM cells express FVIII at the mRNA and protein levels. Reverse transcriptase PCR analysis showed that FVIII mRNA was well expressed in human BM (Figure 1A). Immunofluorescence staining using a polyclonal anti-FVIII antibody, generated in our laboratory, identified FVIII protein in human BM and spleen (see *Online Supplementary Results*). In these organs, FVIII was largely localized in $CD14^+$ cells (Figure 1B,C). This confirmed previous findings of FVIII expression in myeloid cells in the mouse.[23]

We, therefore, explored, in both humans and mice, which hematopoietic cell populations express FVIII

(Figure 2 and *Online Supplementary Figure S2*). Peripheral blood monocytes and monocyte-derived macrophages expressed FVIII at the mRNA and protein levels. However, human monocyte derived-macrophages showed greater FVIII expression than freshly isolated monocytes, as determined by reverse transcriptase PCR (Figure 2A). The FVIII mRNA levels in lymphocytes and neutrophils were very low compared to those in monocytes and macrophages (Figure 2A).

FVIII was not present in hematopoietic cells from HA mice (*Online Supplementary Figure S2A*, lanes 2-4). FVIII mRNA was found only in monocytes of wild-type mice

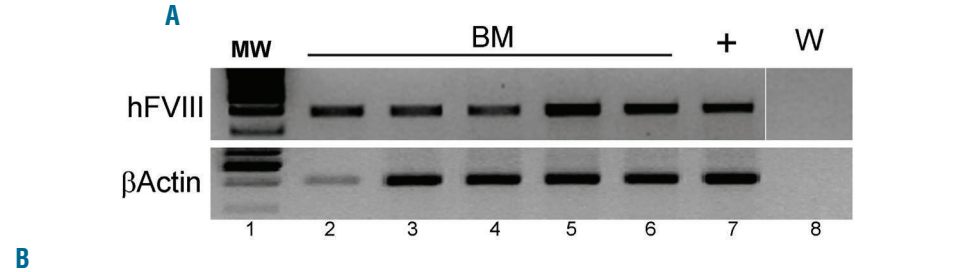

Figure 1. Expression of FVIII in human bone marrow cells. (A) Reverse transcriptase-PCR and (B) immunofluorescence analysis of FVIII expression in five representative human bone marrow samples. A human hepatocellular carcinoma cell line (HepG2) was used as a positive control (+) in the reverse transcriptase-PCR analysis, W (water) and MW (molecular weight). FVIII immunoreactivity was assessed also in CD14+ BM cells (B) and in spleen macrophages (C). Scale bar: 25 μm.

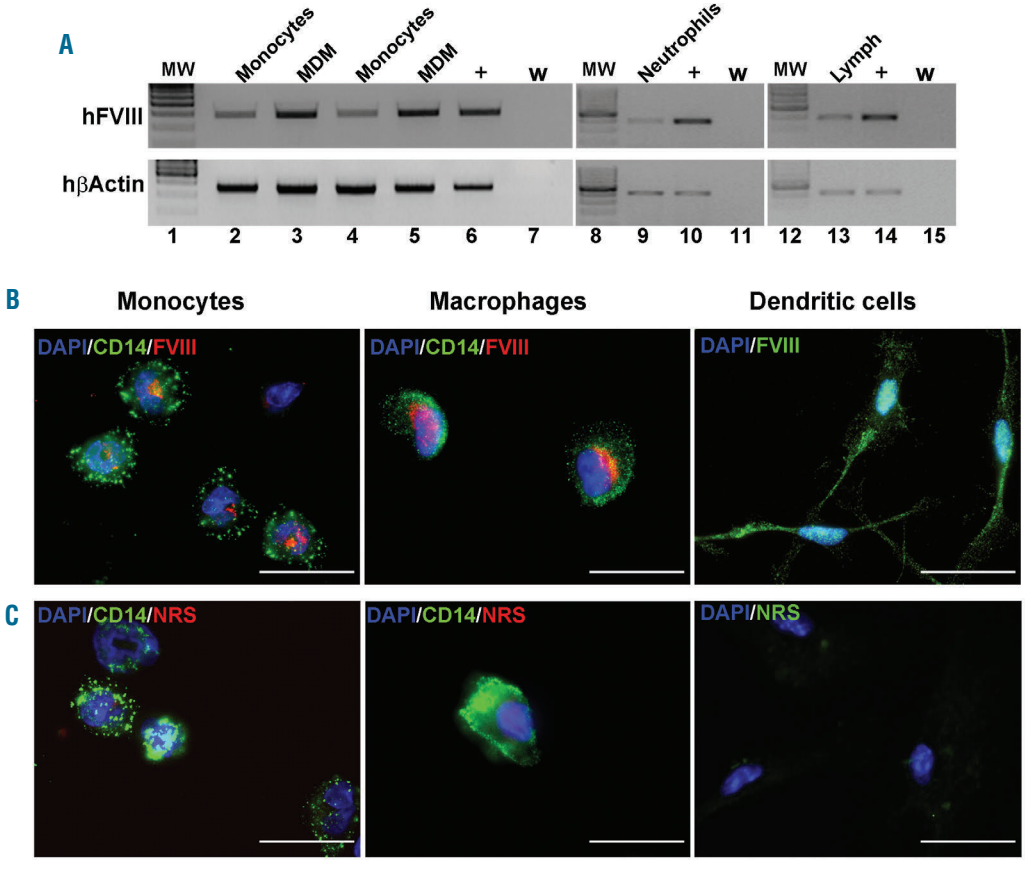

Figure 2. Expression of FVIII in peripheral human blood cells. (A) Reverse transcriptase-PCR analysis of FVIII expression in monocytes, monocyte-derived macrophages (MDM), neutrophils and lymphocytes (lymph). (B) FVIII immunoreactivity in monocytes, macrophages and dendritic cells. (C) Controls were stained with normal rabbit serum (NRS). Scale bar: 25 μm.

(*Online Supplementary Figure S2A*, lanes 5-7). Immunofluorescence demonstrated FVIII in monocytes as well as in monocyte-derived macrophages and dendritic cells with co-staining of FVIII and the CD14 monocyte marker (in human cells) (Figure 2B) or FVIII and the F4/80 monocyte/macrophage marker (in mouse cells) (*Online Supplementary Figure S2B*). Negative controls were incubated with normal rabbit serum (Figure 2C).

Similar results were obtained in differentiated macrophages derived from mouse BM or CD34$^+$ human

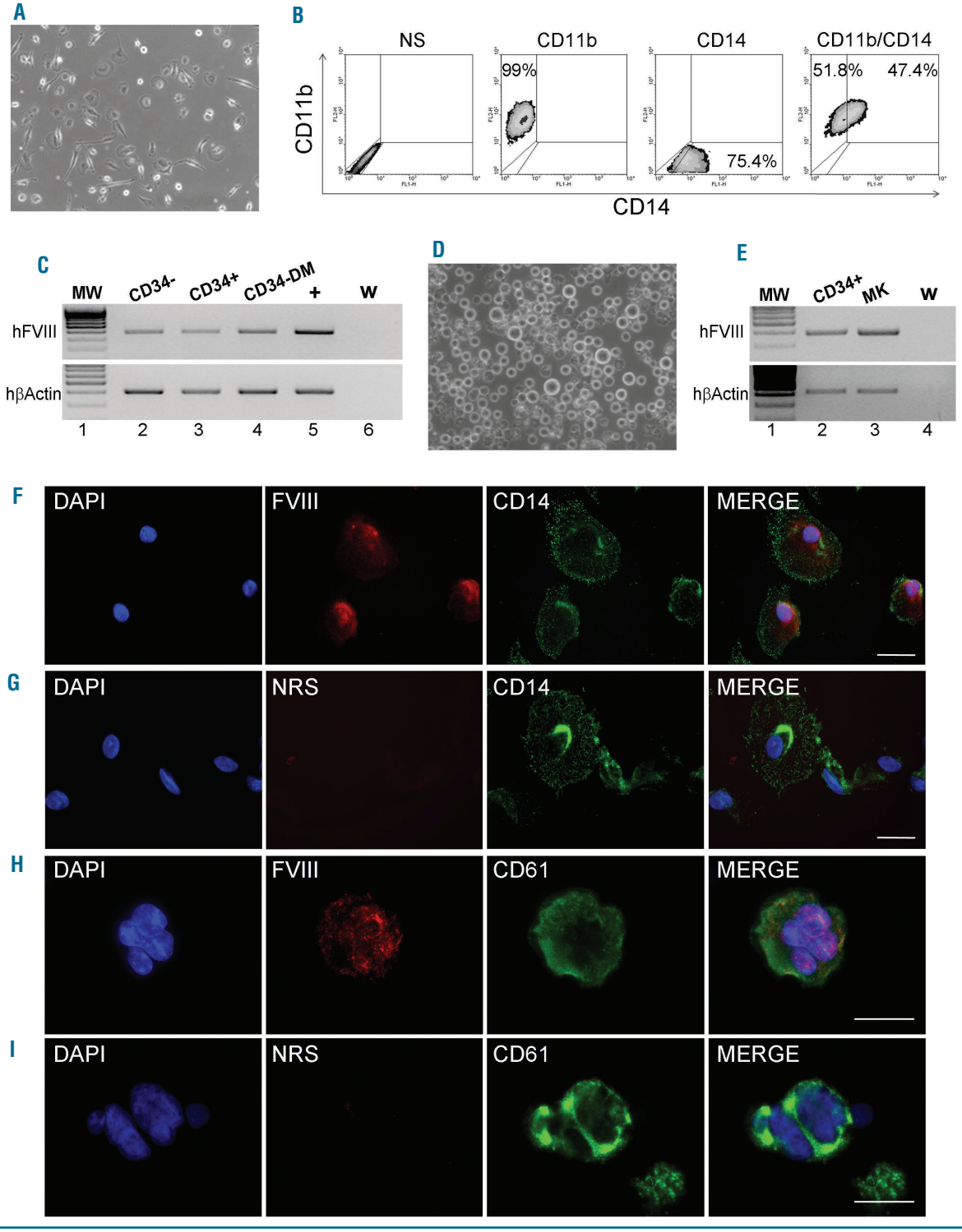

Figure 3. FVIII expression in hematopoietic stem cell-derived macrophages and megakaryocytes. (A, D) Phase contrast microscopy showing CD34$^+$ cells isolated by immunoselection from human cord blood and differentiated into macrophages or megakaryocytes with a cytokine cocktail. (B) Cytofluorimetric analysis of differentiated human macrophages, which expressed the expected markers, CD11b and CD14. (C) PCR on CD34$^+$ cell-derived macrophages and (E) megakaryocytes showing FVIII expression. (F,G) FVIII protein detected by immunofluorecence in CD34$^+$ cell-derived macrophages and (H,I) megakaryocytes. CD34-DM, CD34 derived macrophages; MK, megakaryocytes; NRS, normal rabbit serum. Scale bar: 25 μm.

cord blood cells. After adhesion to dishes, differentiated macrophages displayed typical "fried egg" morphology, abundant cytoplasm and CD11b and CD14 markers (Figure 3A,B and *Online Supplementary Figure S3A*). Reverse transcriptase PCR confirmed that human hematopoietic stem cells and CD34$^+$ cell-derived macrophages expressed FVIII mRNA (Figure 3C). FVIII mRNA was also found in mouse total BM, mainly in c-Kit$^+$ cells as well as in BM-DM (*Online Supplementary Figure S3B*).These results were confirmed by co-staining of FVIII with CD14 in human cells (Figure 3F,G) and of FVIII with F4/80 in mouse cells (*Online Supplementary Figure S3F*).

In view of the physiological role of von Willebrand factor in stabilizing plasma FVIII,[37] we investigated whether megakaryocytes could express and produce FVIII. After human CD34$^+$ or mouse c-Kit$^+$ cells were differentiated into megakaryocytes, cells became larger with rounded morphology (Figure 3D and *Online Supplementary Figure S3C*). Human megakaryocytes expressed FVIII at mRNA (Figure 3E) and protein levels, as shown in immunofluorescent staining by co-expression of FVIII and the specific megakaryocyte marker, CD61 (Figure 3H,I). In mouse megakaryocytes differentiated from c-Kit$^+$ cells expression of von Willebrand factor mRNA and GpαIIβ mRNA was

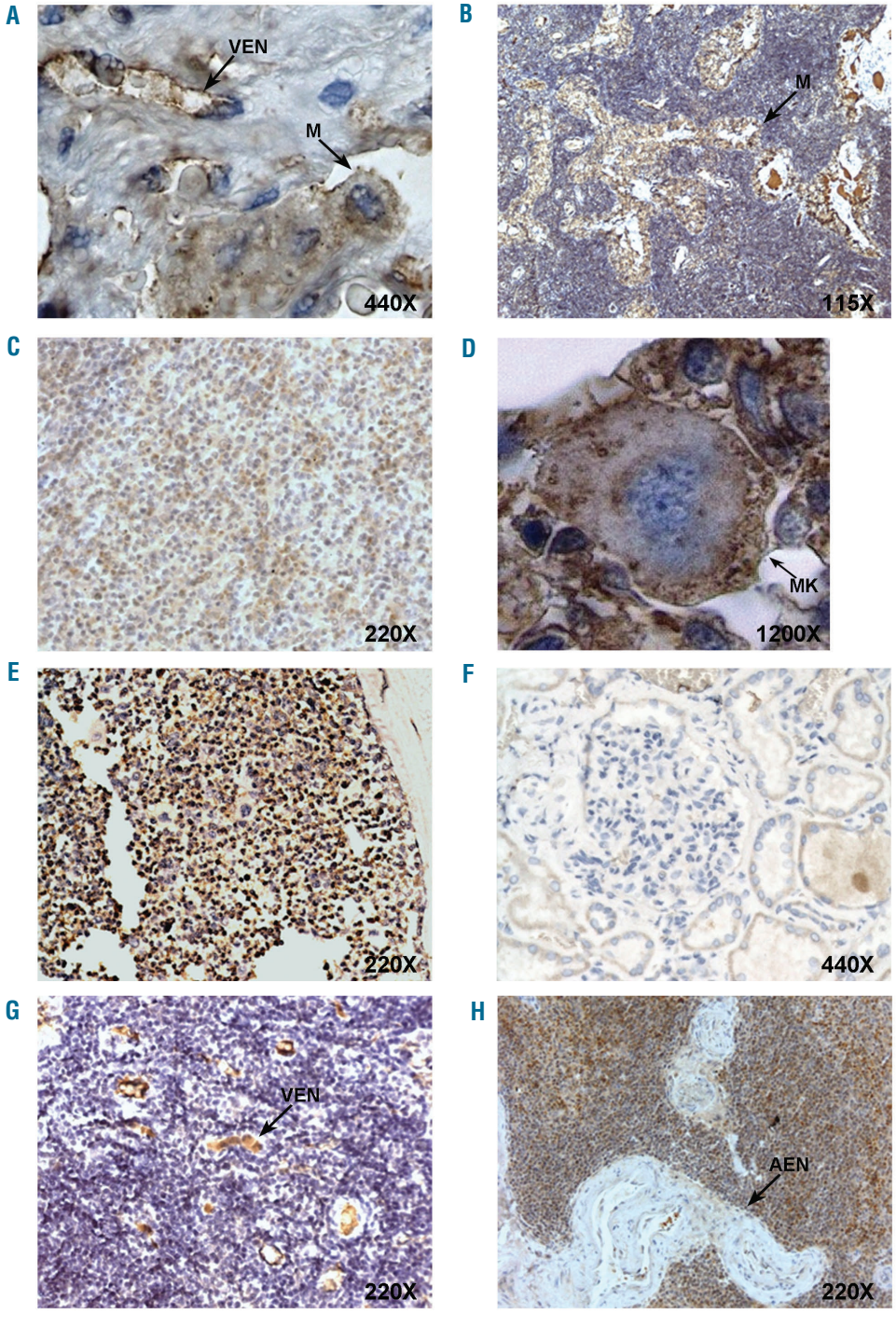

Figure 4. Macrophages in human tissues synthesize FVIII. Immunohistochemical staining with polyclonal anti-FVIII antibodies. FVIII expression is detectable in cells of myeloid origin: (A) macrophages of lung, (B) lymph node, (C) spleen, (D) megakaryocytes and myeloid cells of BM and in (E) myeloid cells of chronic myeloid leukemia. (F) In kidney, glomerular capillaries are negative. (G) Endothelium of post-capillary venules in lymph node is stained, while arterioles and (H) small arteries of spleen are FVIII negative. M: macrophages; MK: megakaryocytes; VEN: venule endothelial cells; AEN: arterial endothelial cells.

up-regulated upon cytokine stimulation *(Online Supplementary Figure S3D)*. FVIII was present in both c-Kit$^+$ cells and megakaryocytes (*Online Supplementary Figure S3E,G*).

Factor VIII expression in human tissues

Immunohistochemistry showed FVIII in the cytoplasm of resident macrophages, especially in pulmonary alveoli (Figure 4A), lymph node sinuses (Figure 4B) and splenic red pulp (Figure 4C). In BM, FVIII was primarily detected in megakaryocytes and myeloid cells (Figure 4D and Figure 1B). In several cases (n=5) of chronic myeloid leukemia, FVIII was present in cells at various stages of differentiation (Figure 4E). Interestingly, FVIII expression in endothelial cells was consistently different in vessels experiencing high or low blood flow with the greater FVIII expression occurring in the latter. For instance, post-capillary venules in lymph nodes, characteristically in areas of T-cell expansion, were strongly positive for FVIII (Figure 4G), whereas renal glomeruli (Figure 4F) and small arteries in splenic white pulp, both exemplifying high pressure flows, were either negative or stained only faintly with FVIII antibody (Figure 4H).

Factor VIII expression in liver cells

Recently, LSEC were shown to be the main source of FVIII in the liver.[38,39] To confirm these findings in human cells, we isolated LSEC, hepatocytes and Küpffer cells from liver biopsies. Immunofluorescence confirmed FVIII expression in cultured LSEC along with co-staining for CD31, CD146 and von Willebrand factor, which are LSEC markers (Figure 5A). Among non-parenchymal cells, FVIII was detected by immunostaining in CD11b$^+$ myeloid cells (Figure 5B-D). Human hepatocytes expressed FVIII, but at a lower level compared to LSEC and not all cells were positive (Figure 5E). We cultured human LSEC and Küpffer cells and verified cell identity by fluorescence activated cell sorting (FACS) using Tie-2 and CD31 for LSEC and CD11b and CD14 for Küpffer cells (Figure 5F). Using quantitative PCR we found that human LSEC contained 5-fold more FVIII mRNA than did hepatocytes or Küpffer cells (P<0.05) (Figure 5G). This was in agreement with immunofluorescence results of FVIII in different types of liver cells (Figure 5A-E). To assess the ability of cells to secrete FVIII, we transplanted 10^7 human hepatocytes (n=9) or 10^7 non-parenchymal liver cells (n=5) mixed with Cytodex3 microcarriers into the peritoneal cavity of NSG-HA mice and then performed aPTT assays after 3 or 7 days to measure plasma FVIII activity. No FVIII activity was detected in recipients of either Cytodex3 micro-carriers alone or hepatocytes at either time points. By contrast, up to 2% plasma FVIII activity was detected in recipients of non-parenchymal liver cells (Figure 5H).

Studies in mice with hemophilia A

Because of the complexity of post-translational modification and trafficking of FVIII in cells, it is crucial to determine whether FVIII-expressing cells can actually secrete the functional protein. To evaluate this property in CD11b$^+$ human monocytes, we transplanted 15x10^6 CD11b$^+$ cord blood-derived cells into NOD-SCID HA mice (n=11) via tail vein injection. One week after transplantation, peripheral blood and spleen contained up to 3% human cells as shown by FACS (Figure 6A,B). Transplanted cells additionally engrafted in the liver, as shown by immunofluorescence staining (Figure 6C). After 3 and 7 days of cell transplantation, in four mice FVIII activity was detected by Coatest assay (Figure 6D). After 1 week, nine out of 11 mice survived tail clip-induced bleeding, whereas all control mice died (Figure 6F). These results were confirmed in NSG-HA mice treated with the same number of CD11b$^+$ cells. In these NSG-HA recipients, after 1 week FACS confirmed the presence of human cells in the spleen and blood (*data not shown*). An aPTT assay also confirmed the appearance of FVIII activity (Figure 6E). In conclusion, human monocytes secreted FVIII and rescued the bleeding phenotype in HA mice.

Transplantation of human CD34$^+$ cells in NSG-HA mice

To determine whether cord blood-derived progenitor cells generated FVIII–expressing cells, we subjected mice to sub-lethal BM conditioning with busulfan (50 mg/kg). Animals were then divided into two groups and transplanted with either 3x10^5 CD34$^+$ cells (n=6, low dose) or 6x10^5 CD34$^+$ cells (n=6, high dose). Human cell chimerism was analyzed 8 and 12 weeks later and cell engraftment was evaluated by FACS for human CD45. Eight weeks after transplantation, CD45$^+$ human cells constituted 39.3±22.7% and 25.3±11.4% of nucleated blood cells, in the low- and high-cell dose group, respectively (Figure 7A,B). Higher cell engraftment was observed in mice receiving the low dose of cells. The engraftment of human CD45$^+$ cells was generally similar over 12 weeks (36.8±16.8% and 25.4±9.1%). FVIII activity was >2% in most cell recipients (Figure 7C), which was sufficient to ameliorate the bleeding phenotype of HA mice. At 12 weeks, higher FVIII activity was detected in the high-cell dose group than in the low-cell dose group (3.7±1.3% *versus* 2.4±1.1%, *P*<0.05 (Figure 7D). After tail clip challenge, nine of the 12 transplanted mice (75%) survived, while all control mice died (Figure 7E). These mice showed FVIII activity of 3.5±1.3% by the aPTT assay and there was a correlation between the phenotypic correction and survival of the animals following tail clip-induced bleeding.

At the end of the tail clip assay, human cell engraftment was evaluated in the spleen and BM. The engraftment of human CD45$^+$ cells was similar in low- and high-cell dose groups (BM: 63.6±6.5% and 54.5±8.5%; spleen: 62.2±17.2% and 62.5±2.3%, respectively) (Figure 8A,B). Myeloid differentiation of transplanted cells was investigated in BM by CD33 and CD14 staining, which showed more CD14$^+$ than CD33$^+$ cells (Figure 8A). In the spleen, transplanted human cells were predominantly B cells; 40% were CD19$^+$, <8% were CD3$^+$ cells and <20% were monocytes, as shown by CD14 and CD11b staining. Engrafted human cells were more often CD11b$^+$ than CD14$^+$ (Figure 8B). No correlation was found between transplanted cell numbers and human cell chimerism, as detected by human CD45$^+$ cells in mouse blood. In mice transplanted with fewer CD34$^+$ cells, more human cells were found and in mice transplanted with more CD34$^+$ cells fewer human cells were observed in the peripheral blood. Transplanted cell engraftment in BM and spleen was similar (Figure 8A, B).

Discussion

The development of new approaches to correct hemophilia A requires insights into cell populations capable of

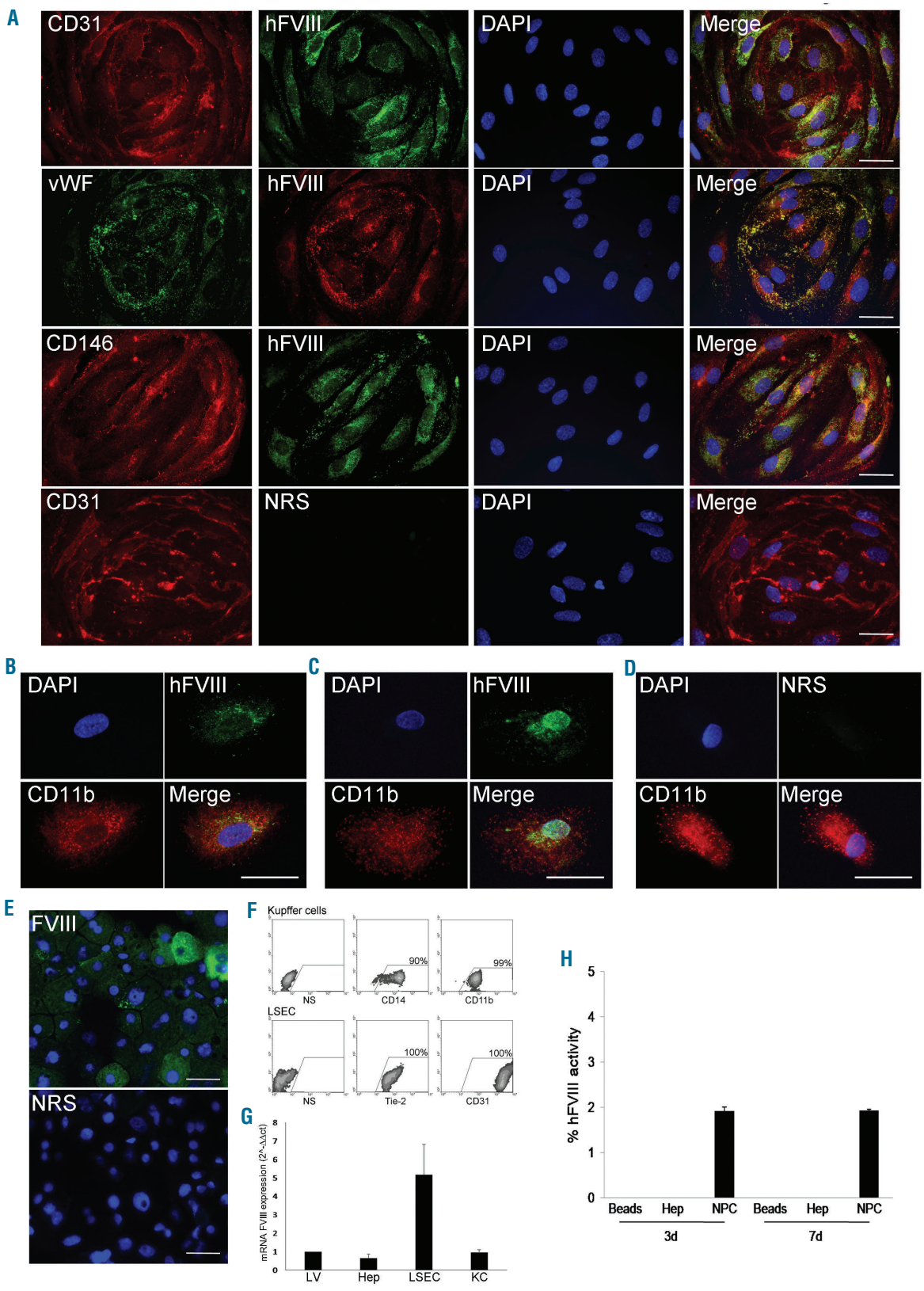

Figure 5. Expression of FVIII in human liver cells. (A-E) Human LSEC, Küpffer cells (KC) and hepatocytes were isolated and FVIII expression was evaluated by immunofluorescence, and (G) quantitative PCR. (F) The identity of isolated LSEC and KC was verified by FACS analysis. (A) Immunofluorescence showing LSEC co-expressing FVIII and liver sinusoidal endothelial cell markers (CD31, CD146 and vWF). (B-D) Immunofluorescence showing FVIII expression in human KC co-stained with CD11b. (E) Immunofluorescence showing FVIII expression in human hepatocytes. (F) FACS analysis demonstrating expression of typical markers by isolated KC ($CD11b^+$ and $CD14^+$) and LSEC ($Tie\text{-}2^+$ and $CD31^+$). (G) Quantitative PCR showing FVIII mRNA expression in human liver and isolated human LSEC, KC and hepatocytes. (H) FVIII activity detected by aPTT assay in plasma from mice following transplantation of human hepatocytes (hep), non-parenchymal cells (NPC) or micro-carriers alone (beads) as control after 3 or 7 days. NRS, normal rabbit serum. Scale bar: 25 μm.

producing FVIII. Early studies suggested that FVIII was expressed mostly in hepatocytes,[40] but other studies found that FVIII was expressed in LSEC and macrophages as well, whereas virtually no signal for FVIII was detected in hepatocytes.[20,41] More recently, we and others established that FVIII is largely produced and secreted by LSEC.[8,9,42,43] Indeed, we were able to correct the bleeding phenotype of HA mice by transplantation of healthy LSEC in the monocrotaline-conditioned liver of hemophilic mice. We also found that HA mice can be treated by reconstitution of healthy BM, indicating that BM–derived hematopoietic or mesenchymal cells can produce and secrete FVIII.[23] This study built on these possibilities by investigating whether human LSEC, hepatocytes or peripheral blood, cord blood and BM cells may be suitable for cell therapy in HA.

Although hepatocytes have been targeted for cell and gene therapy in hemophilia,[44-46] recent studies demonstrated that FVIII is not secreted by hepatocytes, but, on the

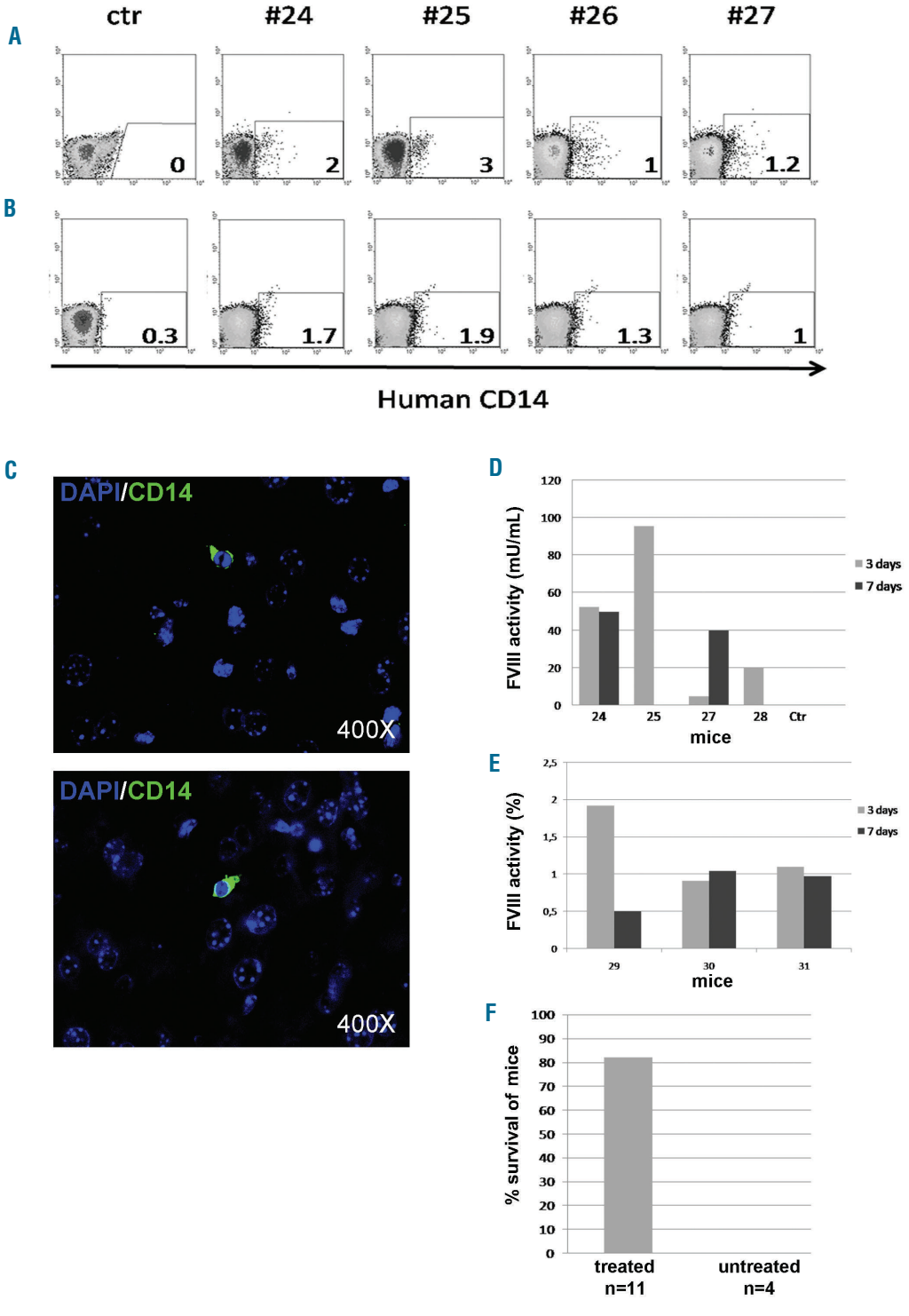

Figure 6. Hemophilia A correction after monocyte injection in hemophilic mice. Identification of transplanted cells in (A) peripheral blood, and (B) spleen, as shown by cytofluorimetric analysis, and (C) in liver of mice as shown by immunofluorescence with anti-CD14 antibodies. (D, E) FVIII activity of treated and control mice measured by chromogenic assay or by aPTT assay 3 and 7 days after $CD11b^{+}$ injection. (F) Survival of monocyte-injected hemophilic A mice after tail clip performed 1 week after treatment. All mice that showed plasma FVIII activity survived tail clipping. Numbers refer to individual mice.

contrary, largely by endothelial cells.[39] In agreement with this, recent studies confirmed the expression of FVIII in endothelial cells using elegant strategies of tissue-specific knocking out of FVIII expression in different cell types using Cre/Lox in transgenic mice. The authors concluded that FVIII is not synthesized in hepatocytes but mainly secreted by the endothelial cells.[10,11] Despite the validity of these data, the fact that the clotting factor assays performed were mainly activity data should be kept in mind since low levels of FVIII secretion by other cell types might still be unidentified. Similarly, tissue analysis in this study showed that FVIII is produced by LSEC and endothelial cells in venous vessels, while FVIII was nearly undetectable in arterial endothelial cells. These findings open up the possibility that high blood flow in arteries plays a role in down-regulating FVIII expression. Additionally, we detected variable levels of FVIII in hepatocytes from healthy human liver, with the clotting factor being absent from

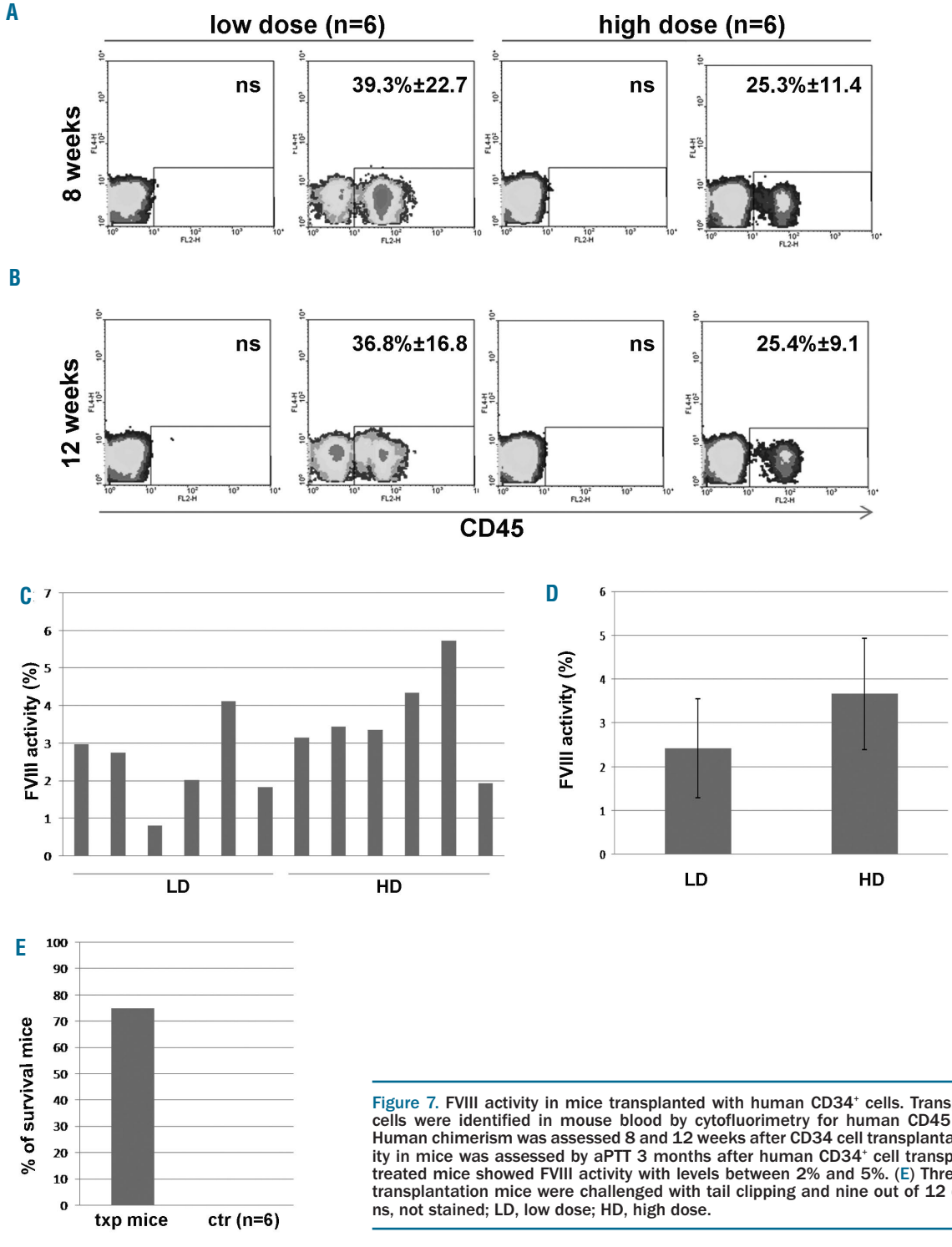

Figure 7. FVIII activity in mice transplanted with human CD34⁺ cells. Transplanted human cells were identified in mouse blood by cytofluorimetry for human CD45 marker. (A, B) Human chimerism was assessed 8 and 12 weeks after CD34 cell transplantation. FVIII activity in mice was assessed by aPTT 3 months after human CD34⁺ cell transplants. (C, D) All treated mice showed FVIII activity with levels between 2% and 5%. (E) Three months after transplantation mice were challenged with tail clipping and nine out of 12 (75%) survived. ns, not stained; LD, low dose; HD, high dose.

some cells. It cannot be excluded that FVIII might have been degraded in these cells. However, in our experience, we have always detected FVIII mRNA in isolated mouse and human hepatocytes. In one study, hepatocyte transplantation under the kidney capsule of HA mice demonstrated transplanted cell survival for up to 100 days with detectable FVIII activity allowing mice to survive after tail clip-induced bleeding,[46] On the other hand, when hepatocytes were transplanted into the peritoneal cavity of HA mice in our study, we were unable to detect correction of plasma FVIII activity. By contrast, when LSEC were transplanted, we readily detected plasma FVIII activity in HA mice. Here, we confirmed these results by showing that peritoneal transplantation of human non-parenchymal liver cells, but not hepatocytes, restored detectable plasma FVIII activity in NSG-HA mice.

In this study, we detected FVIII in myeloid cells, i.e. monocytes/macrophages/dendritic cells, as well as megakaryocytes, including after cell differentiation *in vitro*. Moreover, our survey of hematopoietic organs demonstrated FVIII production in human spleen, BM and lymph nodes, where, besides endothelial cells, FVIII was detected in myeloid cells. Lungs were positive for FVIII expression in endothelial cells and alveolar macrophages. This is in agreement with the findings of van der Kwast and colleagues who found FVIII in pulmonary alveolar macrophages and cells of the splenic red pulp.[20] Interestingly, when monocytes were differentiated in macrophages *in vitro*, the mRNA signal increased indicating that during macrophage differentiation FVIII expression was up-regulated. Similarly, FVIII was expressed in dendritic cells, cord blood-derived cells, as well as lymphocytes, but not in neutrophils. It should be noted that FVIII was originally cloned from T cells and genetic diagnosis of hemophilia in people relies on RNA analysis from lymphocytes.[22] Remarkably, when CD11b$^+$ cells from cord blood were injected into HA mice, we detected FVIII activity in blood for up to 1 week and mice survived after tail clip-induced bleeding. The role of monocytes/macrophages in this process was re-emphasized, since human CD14$^+$ cells

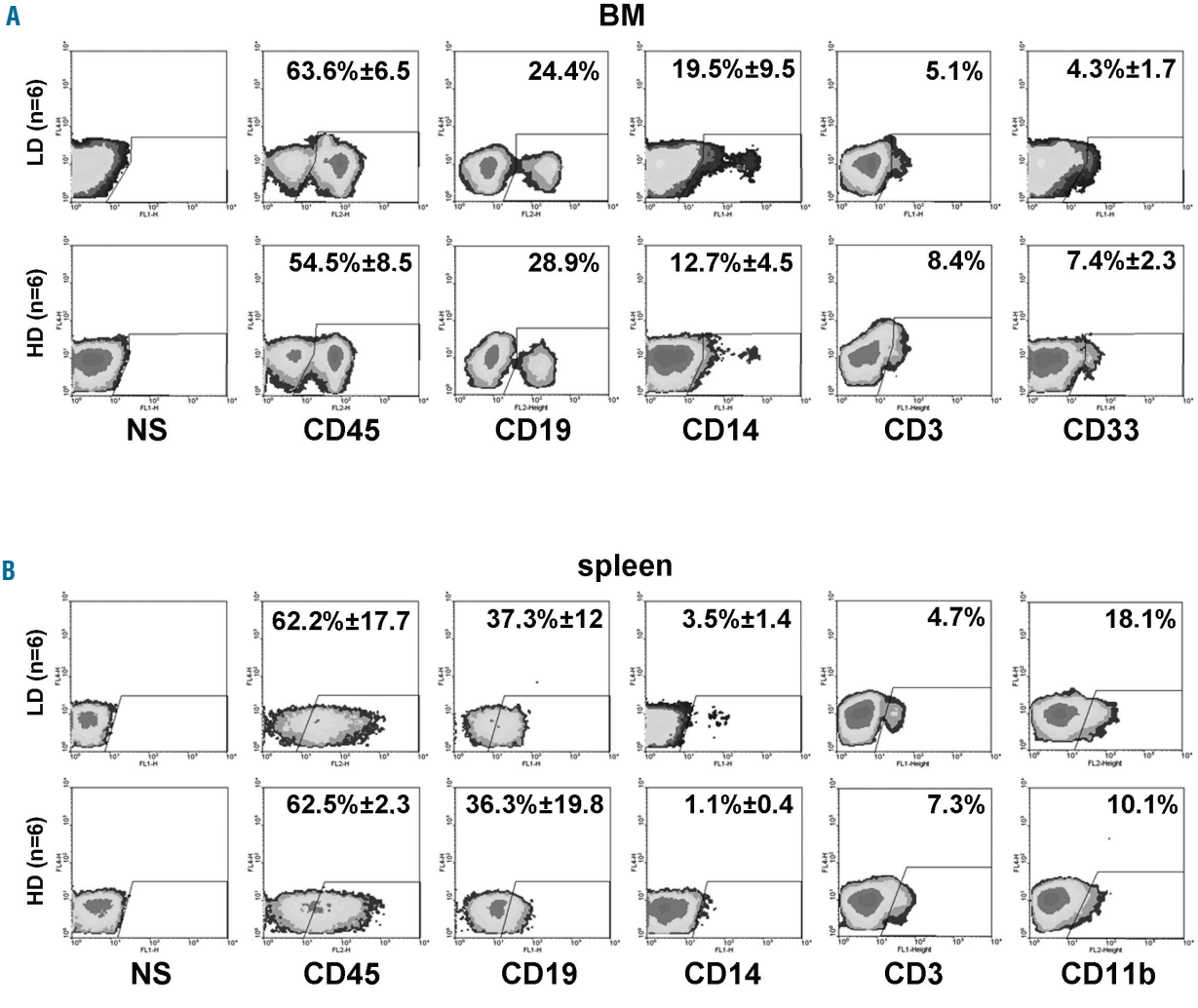

Figure 8. Human chimerism in BM and spleen of transplanted mice. BM and spleens of transplanted mice were stained for human (h) markers 3 months after cell transplantation. (A) BM was stained for hCD45, hCD33 myeloid precursor, hCD14 monocyte marker, hCD3 T-cell marker and hCD19 B cell marker. (B) Spleen was analyzed for the presence of hCD45, hCD14, CD19, hCD3 and CD11b monocyte markers. Percentages without standard deviations are representative of two independent experiments. NS, not stained; LD, low dose; HD, high dose.

were identified in the liver and spleen of transplanted mice. In the case of megakaryocytes, demonstrating the presence of FVIII mRNA was helpful for ensuring that the FVIII originated from endogenous expression, and not simply from uptake of exogenous protein.

FVIII production after hematopoietic stem cell transplantation in humans has been reported but needs more detailed study.[47,48] Our data are in agreement with the report of Caselli and colleagues[47] on an adolescent with hemophilia A who received cord blood hematopoietic stem cell transplantation and improved. The authors described that during follow-up the FVIII levels in the plasma of this patient were increased and that, on the basis of the international classification, the degree of his coagulation defect prior to transplantation qualified him as a severe case whereas after transplant he should be defined as having moderate hemophilia.

Transplantation of cord blood cells has been a successful treatment for BM failure, malignancies, immunological disorders, metabolic diseases, etc. Such applications are attractive because cord blood is widely available and contains greater numbers of early hematopoietic stem/progenitor cells than does BM.[49] As FVIII was expressed in cord blood-derived CD34$^+$ cells, which after transplantation were able to differentiate, secrete FVIII and correct the bleeding phenotype of NSG-HA mice, this should offer further therapeutic opportunities for HA. To increase FVIII expression in HA further, a combined cell–gene therapy approach might be worth envisioning. This should benefit from appropriate gene transfer vectors, including constructs for cell type-specific transcriptional regulation of FVIII expression, as shown by the correction of HA by regulated FVIII expression under a platelet-specific promoter for cell-gene therapy with BM cells.[30,50]

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Authorship and Disclosures

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References

1. Graw J, Brackmann HH, Oldenburg J, Schneppenheim R, Spannagl M, Schwaab R. Haemophilia A: from mutation analysis to new therapies. Nat Rev Genet. 2005;6(6):488-501.
2. Den Uijl IE, Mauser Bunschoten EP, Roosendaal G, et al. Clinical severity of haemophilia A: does the classification of the 1950s still stand? Haemophilia. 2011;17(6):849-853.
3. Dimichele D. Inhibitors: resolving diagnostic and therapeutic dilemmas. Haemophilia. 2002;8(3):280-287.
4. Terraube V, O'Donnell JS, Jenkins PV. Factor VIII and von Willebrand factor interaction: biological, clinical and therapeutic importance. Haemophilia. 2010;16(1):3-13.
5. Storb R, Marchioro TL, Graham TC, Willemin M, Hougie C, Thomas ED. Canine hemophilia and hemopoietic grafting. Blood. 1972;40(2):234-238.
6. Webster WP, Zukoski CF, Hutchin P, Reddick RL, Mandel SR, Penick GD. Plasma factor VIII synthesis and control as revealed by canine organ transplantation. Am J Physiol. 1971;220(5):1147-1154.
7. Madeira CL, Layman ME, de Vera RE, Fontes PA, Ragni MV. Extrahepatic factor VIII production in transplant recipient of hemophilia donor liver. Blood. 2009;113(21):5364-5365.
8. Follenzi A, Benten D, Novikoff P, Faulkner L, Raut S, Gupta S. Transplanted endothelial cells repopulate the liver endothelium and correct the phenotype of hemophilia A mice. J Clin Invest. 2008;118(3):935-945.
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(9):2022-2031.
10. Everett LA, Cleuren AC, Khoriaty RN, Ginsburg D. Murine coagulation factor VIII is synthesized in endothelial cells. Blood. 2014;123(24):3697-3705.
11. Fahs SA, Hille MT, Shi Q, Weiler H, Montgomery RR. A conditional knockout mouse model reveals endothelial cells as the principal and possibly exclusive source of plasma factor VIII. Blood. 2014;123(24):3706-3713.
12. Aronovich A, Tchorsh D, Katchman H, et al. Correction of hemophilia as a proof of concept for treatment of monogenic diseases by fetal spleen transplantation. Proc Natl Acad Sci USA. 2006;103(50):19075-19080.
13. Hollestelle MJ, Thinnes T, Crain K, et al. Tissue distribution of factor VIII gene expression in vivo--a closer look. Thromb Haemost. 2001;86(3):855-861.
14. Jacquemin M, Neyrinck A, Hermanns MI, et al. FVIII production by human lung microvascular endothelial cells. Blood. 2006;108(2):515-517.
15. Kennedy A, Ng CT, Biniecka M, et al. Angiogenesis and blood vessel stability in inflammatory arthritis. Arthritis Rheum. 2010;62(3):711-721.
16. Jiang HC, Gao Y, Dai WJ, et al. Ten-year experience with living related donated splenic transplantation for the treatment of hemophilia A. Transplant Proc. 2006;38(5):1483-1490.
17. Liu L, Xia S, Seifert J. Transplantation of spleen cells in patients with hemophilia A. A report of 20 cases. Transpl Int. 1994;7(3):201-206.
18. Hampton JW, Coussons RT. Splenic transplant for hemophilia. N Engl J Med. 1970;283(1):48.
19. McKee PA, Coussons RT, Buckner RG, Williams GR, Hampton JW. Effects of the spleen on canine factor VIII levels. J Lab Clin Med. 1970;75(3):391-402.
20. van der Kwast TH, Stel HV, Cristen E, Bertina RM, Veerman EC. Localization of factor VIII-procoagulant antigen: an immunohistological survey of the human body using monoclonal antibodies. Blood. 1986;67(1):222-227.
21. Lin Y, Chang L, Solovey A, Healey JF, Lollar P, Hebbel RP. Use of blood outgrowth endothelial cells for gene therapy for hemophilia A. Blood. 2002;99(2):457-462.
22. Wood WI, Capon DJ, Simonsen CC, et al. Expression of active human factor VIII from recombinant DNA clones. Nature. 1984;312(5992):330-337.
23. Follenzi A, Raut S, Merlin S, Sarkar R, Gupta S. Role of bone marrow transplantation for correcting hemophilia A in mice. Blood. 2012;119(23):5532-5542.
24. Yadav N, Kanjirakkuzhiyil S, Kumar S, et al. The therapeutic effect of bone marrow-derived liver cells in the phenotypic correction of murine hemophilia A. Blood. 2009;114(20):4552-4561.
25. Ide LM, Iwakoshi NN, Gangadharan B, et al. Functional aspects of factor VIII expression after transplantation of genetically-modified hematopoietic stem cells for hemophilia A. J Gene Med. 2010;12(4):333-344.
26. Moayeri M, Hawley TS, Hawley RG. Correction of murine hemophilia A by hematopoietic stem cell gene therapy. Mol Ther. 2005;12(6):1034-1042.
27. Neyman M, Gewirtz J, Poncz M. Analysis of the spatial and temporal characteristics of platelet-delivered factor VIII-based clots. Blood. 2008;112(4):1101-1108.
28. Ohmori T, Mimuro J, Takano K, et al. Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Ibalpha promoter: in vivo model for platelet-targeting gene therapy. FASEB J. 2006;20(9):1522-1524.
29. Shi Q, Wilcox DA, Fahs SA, et al. Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. J Thromb Haemost. 2007;5(2):352-361.
30. Shi Q, Wilcox DA, Fahs SA, et al. Factor VIII

ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. J Clin Invest. 2006;116(7):1974-1982.
31. Doering CB, Gangadharan B, Dukart HZ, Spencer HT. Hematopoietic stem cells encoding porcine factor VIII induce procoagulant activity in hemophilia A mice with pre-existing factor VIII immunity. Mol Ther. 2007;15(6):1093-1099.
32. Ramezani A, Hawley RG. Correction of murine hemophilia A following nonmyeloablative transplantation of hematopoietic stem cells engineered to encode an enhanced human factor VIII variant using a safety-augmented retroviral vector. Blood. 2009;114(3):526-534.
33. Ramezani A, Zweier-Renn LA, Hawley RG. Factor VIII delivered by haematopoietic stem cell-derived B cells corrects the phenotype of haemophilia A mice. Thromb Haemost. 2011;105(4):676-687.
34. Wilcox DA, Shi Q, Nurden P, et al. Induction of megakaryocytes to synthesize and store a releasable pool of human factor VIII. J Thromb Haemost. 2003;1(12):2477-2489.
35. Shi Q, Fahs SA, Kuether EL, Cooley BC, Weiler H, Montgomery RR. Targeting FVIII expression to endothelial cells regenerates a releasable pool of FVIII and restores hemostasis in a mouse model of hemophilia A. Blood. 2010;116(16):3049-3057.
36. Ito M, Hiramatsu H, Kobayashi K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood. 2002;100(9):3175-3182.
37. Weiss HJ, Sussman, II, Hoyer LW. Stabilization of factor VIII in plasma by the von Willebrand factor. Studies on posttransfusion and dissociated factor VIII and in patients with von Willebrand's disease. J Clin Invest. 1977;60(2):390-404.
38. Fomin ME, Zhou Y, Beyer AI, Publicover J, Baron JL, Muench MO. Production of factor VIII by human liver sinusoidal endothelial cells transplanted in immunodeficient uPA mice. PLoS One. 2013;8(10):e77255.
39. Shahani T, Covens K, Lavend'homme R, et al. Human liver sinusoidal endothelial cells but not hepatocytes contain factor VIII. J Thromb Haemost. 2014;12(1):36-42.
40. Zelechowska MG, van Mourik JA, Brodniewicz-Proba T. Ultrastructural localization of factor VIII procoagulant antigen in human liver hepatocytes. Nature. 1985;317(6039):729-730.
41. Stel HV, van der Kwast TH, Veerman EC. Detection of factor VIII/coagulant antigen in human liver tissue. Nature. 1983;303(5917):530-532.
42. Hellman L, Smedsrod B, Sandberg H, Pettersson U. Secretion of coagulant factor VIII activity and antigen by in vitro cultivated rat liver sinusoidal endothelial cells. Br J Haematol. 1989;73(3):348-355.
43. Do H, Healey JF, Waller EK, Lollar P. Expression of factor VIII by murine liver sinusoidal endothelial cells. J Biol Chem. 1999;274(28):19587-19592.
44. Connelly S, Mount J, Mauser A, et al. Complete short-term correction of canine hemophilia A by in vivo gene therapy. Blood. 1996;88(10):3846-3853.
45. McIntosh J, Lenting PJ, Rosales C, et al. Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. Blood. 2013;121(17):3335-3344.
46. 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(1):132-140.
47. Caselli D, Morfini M, Paolicchi O, Frenos S, Casini T, Arico M. Cord blood hematopoietic stem cell transplantation in an adolescent with haemophilia. Haemophilia. 2012;18(2):e48-49.
48. Ostronoff M, Ostronoff F, Campos G, et al. Allogeneic bone marrow transplantation in a child with severe aplastic anemia and hemophilia A. Bone Marrow Transplant. 2006;37(6):627-628.
49. Liao Y, Geyer MB, Yang AJ, Cairo MS. Cord blood transplantation and stem cell regenerative potential. Exp Hematol. 2011;39(4):393-412.
50. Shi Q, Kuether EL, Chen Y, Schroeder JA, Fahs SA, Montgomery RR. Platelet gene therapy corrects the hemophilic phenotype in immunocompromised hemophilia A mice transplanted with genetically manipulated human cord blood stem cells. Blood. 2014;123(3):395-403.