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Therapeutic potential of fetal liver cells transplantation in hemophilia A mice

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Contributions

SM, SA and MJS designed and performed experiments and analysed data. SM, SA and AC conducted the in vivo studies. LJS and AL performed the mRNA analysis. EB, VK, TGL, RG and CB set up immunofluorescence and flow cytometry analysis. AF and MJS conceived the study. AF generated most funding, supervised the whole project and analysed data. SM, SA, MJS and AF drafted the paper that was completed by all authors that critically reviewed the manuscript and approved the final version.

Disclosures

Nothing to disclose.

Data-sharing statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

ABSTRACT

Hemophilia A (HA) cell therapy approaches in pediatric individuals require suitable factor (F)VIII-producing cells for stable engraftment. Liver sinusoidal endothelial cells (LSEC) and hematopoietic stem cells (HSC) have been demonstrated to be suitable for the treatment of adult HA-mice. However, after transplantation in busulfan (BU)-conditioned newborn mice, adult LSEC/HSC cannot efficiently engraft, while murine fetal liver (FL) hemato/vascular cells from embryonic day 11-13 of gestation (E11-E13), strongly engraft the hematopoietic and endothelial compartments while also secreting FVIII. Our aim was to investigate the engraftment of FL cells in newborn HA mice for obtaining a suitable "proof of concept" for the development of a new HA treatment in neonates. Hence, we transplanted FLE11 or E13 cells and adult bone marrow (BM) cells into newborn HA mice with or without BU preconditioning. The engraftment levels and FVIII activity was assessed starting from 6 weeks after transplantation. FLE11-E13+BU-transplanted newborns reached up to 95% engraftment with stable FVIII activity levels observed for 16 months. FLE13 cells showed engraftment ability even in absence of BU preconditioning, while FLE11 cells did not. BM+BU transplanted newborn HA mice showed high levels of engraftment; nevertheless, in contrast to FL cells, BM cells cannot engraft HA newborns in non-conditioning regimen. Finally, none of the transplanted mice developed anti-FVIII antibodies. Overall, this study sheds some light on the therapeutic potential of healthy FL cells in the cure of HA neonatal/pediatric patients.

INTRODUCTION

Spontaneous haemorrhagic events occurring in hemophilia A (HA) patients are caused by a reduced or absent coagulation FVIII activity[1]. Presently, these bleeding events are managed by replacement therapy compelling the patients to frequent infusions of exogenous FVIII as prophylaxis[2–4], with the short FVIII half-life (~10-12 hours) and high costs of the treatment representing the major drawbacks. Moreover, approximately 30% of severe HA patients develop anti-FVIII neutralizing antibodies (inhibitors), thus reducing or nullifying the effectiveness of the replacement therapy[5]. New therapeutic approaches rapidly evolved in the last decade as extended half-life FVIII concentrates, FVIII mimetics (bi-specific antibodies), and molecules targeting natural anti-coagulant pathways (e.g. Fitusiran)[6,7]. However, all these strategies exert only a temporary therapeutic effect, while cell and/or gene therapy approaches aim to the "one-time treatment" able to induce long term correction with sustained FVIII expression in HA patients[6,7]. Indeed, ongoing phase 1/2 and phase 3 AAV liver directed FVIII gene therapy clinical trials (NCT03734588, NCT03588299, NCT03003533, NCT04370054, NCT03370913) have been showing promising results for the treatment of adult HA patients[8,9]. More recently, the European Commission has granted the conditional marketing authorization to ROCTAVIAN™ gene therapy for the treatment of adult patients affected by the severe form of hemophilia A (https://www.ema.europa.eu/en/medicines/human/orphan-designations/eu-3-16-1622, https://investors.biomarin.com/2022-08-24-First-Gene-Therapy-for-Adults-with-Severe-Hemophilia-A,-BioMarins-ROCTAVIAN-TM-valoctocogene-roxaparvovec-,-Approved-by-European-Commission-EC). However, several difficulties remain, including lack of viral vector infection specificity, pre-existing immunity to viral vectors, and the inability to insert the full-length F8 gene due to restrictive viral cargo sizes. Additionally, all the above-mentioned clinical trials have included only adult HA patients, while none has involved paediatric patients which could not be optimal candidates for these approaches because of the AAV non-integrating nature and consequent transgene dilution during the physiological liver growth[10]. For these reasons additional studies and possibly alternative approaches are required for the treatment of early age/neonate HA patients.

Several studies demonstrated that FVIII is produced by endothelial cells, mainly liver sinusoidal endothelial cells (LSECs)[11–17] and to a lesser extent by bone marrow (BM)-

derived hemopoietic and mesenchymal cells[18–21], that showed stable cell engraftment and sustained production of therapeutic FVIII levels following transplantation in preclinical HA murine models.

Following transplantation, the efficiency of vascular and hematopoietic engraftment depends on the transplantation procedure and the pre-conditioning regimen of the recipient. Transplanted adult LSECs require pre-conditioning regimens using toxic drugs/compounds (e.g. monocrotaline, MCT), partial hepatectomy or irradiation[12,22] that are not suitable for the treatment of HA patients and would be even more damaging for pediatric individuals. Thus, less harmful pre-conditioning regimens with possibly lower/minor and well-defined side effects might represent a valid and more acceptable alternative. One possible treatment is represented by the pre-conditioning with Busulfan (BU), used in murine models[23–25] and in clinic with both pediatric and adult patients[26,27]. BU induces myeloablation and besides, as a side effect, damages the vascular endothelium[28], thus possibly promoting both hematopoietic and endothelial cell engraftment in experimental models.

Efficiency of cell engraftment is also dependent on the donor and recipient age, as shown for different tissues[29], which represents a relevant issue in a pediatric context. In fact, it has been previously shown milder engraftment potential of adult LSECs and adult (A)BM-derived HSC compared to FL LSECs when transplanted to MCT-conditioned adult recipient mice[30]. In general, FL cells seem to possess stronger repopulation activity compared to adult HSCs[31–33] and LSEC[23], thus constituting a potential source for cell therapy approaches.

To harness the potential of novel cell types for HA therapeutic purposes in neonatal individuals, it is central that transplanted cells engraft efficiently and differentiate into long term repopulating FVIII-producing cells. We previously reported that different percentages of HSCs and endothelial progenitor cells populations from the FL are capable to reconstitute the hematopoietic and liver endothelial compartments when transplanted into BU conditioned newborn mice or adult irradiated recipient mice[23,24]. However, whether healthy mouse FL cells can engraft, proliferate and reconstitute the hemato/vascular compartment of newborn HA mice is still unknown. Furthermore, it remains to be established whether these cells will assure long-term FVIII production and secretion at therapeutic levels. Here, using healthy FL cells in a preclinical neonatal murine model of HA we determined the conditions and a rational method to establish a novel cell therapy approach for the treatment of HA pediatric patients,

supporting the potential of FL cells as source of FVIII production and establishing the "proof of concept" that cell therapy can be used in pediatric hemophilic patients.

METHODS

Animals

Animal studies were approved by the Animal Care and Use Committee of the Università del Piemonte Orientale "A. Avogadro" (Novara, Italy) and the by the Italian Ministry of Health with authorization n. 758/2021-PR, and the Ethical Review Board of the Universidad Pablo de Olavide (Seville, Spain) according to the EU regulations. In vivo experiments were performed on recipient newborn and adult hemophilia A mice in C57BL/6 background (C57BL/6-HA)[18]. Donor FL and BM cells were isolated from GFP+ mice in C57BL/6 background (C57BL/6-Tg(ACTbEGFP)1Osb/J, Strain #:003291)[18]. FL cells were obtained from embryonic day 11 (E11) or E13 of gestation[23,24]. Timed breedings of GFP+ transgenic mice were established to obtain the fetuses. Vaginal plugs were checked daily and the day the plug was detected considered as E0. To generate recipient conditioned newborn mice, HA pregnant females were treated with busulfan (15.5mg/kg; Sigma-Aldrich) plus 1U recombinant human (rh) FVIII (ReFacto®, Pfizer) 48hrs and 24hrs (BU group) or 24 hrs (1/2BU group) before delivery, while adult mice (8 weeks old) received BU injections (30 mg/kg/injection) 48h and 24h before transplantation.

Cell isolation and transplantation

Adult BM, FLE11 and E13 cells isolation and transplantation into newborns and adult HA mice were performed as previously described[18,24]. Briefly, fetuses were harvested from GFP+ females from day 11 (E11) to E13 of gestation. Fetal livers were dissected and transferred individually into ice-cold D-PBS with Ca^{2+} and M^{g2+} (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS) (Euroclone). Fetal liver cells were isolated by mechanical disaggregation. Only fetal liver samples presenting GFP+ cells by flow cytometry analysis (FACSCalibur, BD) were pooled and resuspended in D-PBS 1% FBS. Adult bone marrow cells were flushed from tibias and femurs of 6 weeks old GFP+ mice, treated with red blood lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), washed and resuspended in D-PBS 1% FBS. 0.3-5x10^6 cells were resuspended in 50 µl of D-PBS 1% FBS containing 0.2U rh FVIII and

injected over 30 seconds in the superficial temporal vein or facial vein of day 2 HA newborn mice, while for adult mice cells were resuspended in 300 µl and injected into the tail vein. The procedure or the injected volume did not cause adverse effects or severe harm to the recipient mice.

FVIII activity, tail clip assay and ELISA

FVIII activity was measured on the plasma using the activated partial thromboplastin time (aPTT) as previously described[34]. Standard curves were generated by serially diluting plasma pooled from GFP+ mice into HA pooled mouse plasma. Presence of anti-FVIII antibodies in plasma of treated mice was evaluated by indirect ELISA as previously described[34,35]. The tail clip assay was performed as previously described[34].

Flow Cytometry Analysis

Cells from peripheral blood and organs were prepared as previously described[36]. Liver nonparenchymal cells (NPCs) were isolated after liver perfusion as previously published[12]. Samples were stained with antibodies listed in Supplementary table 1. Samples were acquired on the Attune NxT Acoustic Focusing Cytometer (Thermofisher Scientific) and analysis was performed by FlowJo (Tree Star Inc.).

Immunofluorescence.

The organs harvested from treated mice were processed as previously described[34,37]. Cryostat sections of 4-µm thickness were blocked in blocking buffer (5% goat serum, 1% BSA, 0.1% Triton X-100 in PBS), incubated with primary antibodies at RT, and then incubated in the dark at RT with the secondary antibody along with DAPI. Sections were finally mounted with Mowiol mounting media (Sigma-Aldrich) and observed under fluorescence microscope (LEICA DM5500B) and Leica Application Suite X (LAS X) software.

RNA Isolation and qRT-PCR for F8

For quantitative real-time polymerase chain reaction (qRTPCR), total RNA was extracted and cDNA obtained as previously described[38]. Results were analysed using the relative expression method ($2^{-\Delta Ct}$). The PCR primers designed for mouse F8 and GAPDH are: Mouse F8 E16 F 5' TGGCACCCACAGAAGATGAG 3' and Mouse F8 E17 R 5' GGCAAATCAGAAGGGGTCCA 3' (amplicon size 108 bp); GAPDH F 5'

CATGGCCTTCCGTGTTCCTA 3' and GAPDH R 5' GCGGCACGTCAGATCCA 3' (amplicon size 55 bp).

Statistical analysis

The statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software). Data were analysed for normal distribution of population with D'Agostino-Pearson omnibus normality test followed by a 1-way analysis of variance (1way ANOVA). 2-way ANOVA followed by a post hoc Bonferroni's test was run to compare the engraftment between groups. Pearson's correlation test was performed to correlate percentage of engraftment and FVIII activity in all mice. Statistical significance was assumed for $p < 0.05$.

RESULTS

Engraftment of FL cells in HA newborn recipient mice contributes to long-term FVIII production at therapeutic levels.

To determine whether engraftment of FL cells into HA neonates could ameliorate the bleeding phenotype, we injected FL cells from congenic GFP+ mice into the facial vein of BU-treated newborn HA mice (Figure 1A). We transplanted different numbers of cells from E11 or E13 FL according to the developmental stage (Table 1)[23,24]. After transplantation, mice were periodically monitored for engraftment (GFP+ cells in peripheral blood [PB]) and plasma FVIII activity. Flow cytometry analysis showed GFP+ cells in PB of all mice receiving FL cells with BU preconditioning up to 16 months (Figure 1B). Moreover, the FLE13 group displayed significantly higher chimerism than the FLE11 mice, starting from12 months after transplantation.

Along with the engraftment we evaluated the mouse FVIII (mFVIII) activity: all transplanted mice showed FVIII activity >5% (Figure 1C and Table 1) without anti-FVIII antibodies production (Figure 1D). Following transplantation, percentage of PB GFP+ cells and FVIII activity showed a direct significant correlation (Pearson's correlation test, $p<0.0001$) (Supplementary Figure 1), suggesting that FVIII production correlated to the PB engraftment level. The correction of bleeding phenotype in treated mice was evaluated by tail clip challenge at 16 months after transplantation, showing a significant reduction in blood loss in transplanted mice

compared to control BU-treated HA mice (noFL+BU) (Figure 1E). This indicates that engraftment of FL cells in HA newborn recipient mice can contribute to a lifelong correction of the HA bleeding phenotype.

FL-derived hematopoietic cells are responsible for long term FVIII production in transplanted mice.

To address the characteristics of engrafted cells, GFP+ cells were analyzed in spleen, BM and liver. Flow cytometry and immunofluorescence analysis showed that GFP+ cells from the spleen and BM of recipient mice were mainly of hematopoietic origin (CD45+) in both FLE11 and FLE13 groups in accordance with the percentage of GFP+ cells in PB (Figure 2A-E) 16 months after transplantation.

As FL cells showed the ability to reconstitute liver endothelial cells and LSECs[13,23], we performed flow cytometry analysis on hepatic non parenchymal cell (NPCs) fraction and immunofluorescence on liver sections from FLE11 and FLE13 transplanted mice. The data showed the presence of GFP+ cells in the liver of all treated mice, where hepatic NPC GFP+ cells were mainly hematopoietic cells (CD45+), while only a low percentage of liver CD31+CD45- endothelial cells/LSECs showed GFP expression (Figure 2B&C).

Immunostaining on spleen and liver sections from these mice confirmed long-term contribution of FL cells to hematopoietic cells (CD45+) and low contribution to endothelial cells (CD31+) in the liver (Figure 2D&E). In accordance to flow cytometry and immunofluorescence data, F8 mRNA was mainly detected in spleen, BM and liver NPC (Figure 2F). Altogether, these data confirm the potential of FL cells to engraft in newborn HA mice pre-treated with BU.

Effect of busulfan dosage on FL cells engraftment in newborn HA mice.

Since we observed high engraftment (up to 95%) in mice with BU-pretreatment, we evaluated the possibility of obtaining high engraftment levels while reducing preconditioning regimen. We thus repeated the transplantation studies in 2 groups of newborn HA mice, one pre-treated with the standard dose of BU (BU2x) while the second group received half dosage (BU1x). Pre-treated newborns were transplanted with FLE11 or FLE13 cells as described above, and engraftment level was regularly evaluated. Six months after transplantation both BU1x groups showed significantly lower engraftment compared to their BU2x counterparts with the FLE11 cells-injected

mice displaying the lowest level (~2.5%) (Figure 3, Table 1, and Supplementary Table 2). As described previously, plasma mFVIII activity correlated with engraftment levels and differences observed between mice receiving the same FL cells but different BU dosage as well as between mice treated with BU1x and transplanted with FLE13 and FLE11 were statistically significant. Therefore, the higher levels of engraftment and therapeutic levels of FVIII were achieved following the full BU treatment regimen.

Bone marrow cell transplantation in newborn HA mice.

We previously showed that the transplantation of adult BM (ABM) cells can correct the bleeding phenotype of adult HA mice[18]. On this basis, we decided to evaluate the ability of ABM cells to engraft and correct the bleeding phenotype of newborn HA mice. Following transplantation in newborn HA mice, we evaluated the engraftment and the phenotypic correction at 6 weeks, 3 months and 6 months and we compared these results with those obtained with mice receiving FLE13 cells. Mice transplanted with FL cells showed engraftment significantly higher than mice transplanted with BM at 6 weeks (FL 79.5%±4.5% vs BM 68.53%±5.9%) and 6 months (FL 90.2%±2.6% vs BM 74.9%±6.6%) after transplantation ($p<0.05$) (Figure 4A); however, 6 weeks after transplantation mice receiving BM cells showed marginally higher mFVIII activity than mice transplanted with FLE13 (11.8%±3.6 FL vs 14.7%±4.9 BM; $p<0.05$), while FLE13 transplanted mice showed higher mFVIII activity compared to mice transplanted with BM cells after 6 months (14.3%±3.8 FL vs 12.7%±3 BM; $p<0.05$) (Figure 4B). Interestingly, in our control mice not treated with busulfan (noBU), we observed engraftment in mice receiving FLE13 cells but not in mice transplanted with ABM cells (~15% FLE13 versus [vs] <1% BM; $p < 0.01$) (Figure 4C), and murine FVIII activity levels correlating with engraftment levels, showing to be significantly higher when compared with ABM cells (6.3% FLE13 vs <0.1% BM; $p<0.01$) (Figure 4D). Levels of murine FVIII activity were additionally confirmed by bleeding assay (Supplementary Figure 2). These data confirm that the correction of bleeding phenotype using ABM cell transplantation requires BU preconditioning in newborn HA mice, while FLE13 showed long term engraftment potential and mFVIII production ability even in absence of BU preconditioning.

FL cells engraftment in adult HA mice requires BU pre-treatment.

It has been previously shown that FLE9.5-10.5 cells showed preferential engraftment in neonatal mice, while HSC from later embryonic developmental stage or from adult BM showed higher engraftment activity in adult mice[31].

Thus, we evaluated the engraftment ability of FLE13 in adult (8 weeks old) HA mice with or without sublethal myeloablation. After transplantation, mice receiving FL cells without BU preconditioning showed no engraftment, while we observed only engraftment in BU pretreated mice. Six weeks after transplantation, GFP+ cells in peripheral blood ranged from 50 to 70%, while after six months GFP+ cells were more than 90% (Figure 5A), with a concomitant bleeding phenotype correction ranging from ~9% at 6 weeks to 20% after 6 months (Figure 5B), data additionally confirmed by tail clip assay (Supplementary Figure 3).

These data indicate that correction of the bleeding phenotype of adult HA mice requires preconditioning treatments to allow the engraftment of FL cells.

DISCUSSION

Over the last two decades many attempts have been made to develop a long-term treatment for HA by using cell and/or gene therapy strategies. Despite of numerous studies on adults, less data are currently available on newborn HA mice following cell and gene therapy[6,8,39].

New cell therapy approaches able to ensure a stable long-term FVIII production at therapeutic levels are extremely attractive, especially for the treatment of early age/neonate patients. Selection of the right cell type for transplantation is the key element in a cell-based therapy approach. Endothelial cells, mainly LSECs are the principal source of FVIII within the body[12,16,40]; additionally, hematopoietic cells were able to produce and secrete FVIII, although to a lesser extent compared to endothelial cells, thus correcting the bleeding phenotype of adult recipient mice[18,19].

Neonatal recipients are more permissive for embryonic/early fetal hematopoietic progenitor cells engraftment[31,41] and higher proliferative activity of fetal-derived hematopoietic progenitors also confers increased engraftment potential on newborns as well as in adult recipients[31,33]. Taking these previous studies in consideration, we investigated in a murine preclinical model the FL cells potential as source for the

development of a cell therapy approach to treat pediatric HA patients. We previously demonstrated that FL cells engrafted and repopulated the hemato/vascular compartment of wild type (wt) newborn recipient mice[24], and additionally characterized in the FL a unique cell population capable of a stable multiorgan endothelial reconstitution, mostly composed of endothelial committed cells[23]. More recently, we also reported that FVIII mRNA progressively increases in the FL and in other different embryonic locations from day 9 to day 12 of gestation (E9-12), in parallel to the expansion of the vascular network[38].

In this study, following transplantation in newborn HA mice, both FLE11 and FLE13 cells showed long term engraftment potential[23,24], and were able to correct the bleeding phenotype of the recipient hemophilic mice. These results are in line with the ones obtained in lethally irradiated adults HA mice, in which we observed a long-term phenotypic correction following total BM cells transplantation[18].

Since BM is a readily available clinical source for cell therapy[19,27], we transplanted BM cells into newborn HA mice. Previous studies showed that FL cells exhibited a higher capacity for long-term and multilineage hematopoietic reconstitution than equal numbers of BM cells transplanted into lethally irradiated adult mice[31,33] or into newborn recipients[31]. Similarly, in our settings FLE13 cells engraftment in neonate HA mice was higher compared to ABM cells, although the difference was not statistically significant at all time points. Despite lower engraftment, BM cells-transplanted mice showed higher mFVIII activity up to 4 months. Analysis of the lineage output of transplanted HSCs through development has previously revealed a trend for reduced myeloid lineage output from FL13 compared to adult BM HSCs at 4 months post-transplant in irradiated adult recipient mice[42]. Considering that myeloid cells are the main producers of FVIII among the hematopietic lineage[19], this data can support the notion that BM cells may better contribute to myeloid compartment and hence to FVIII production in newborns. Further work is necessary to characterize any difference in engraftment and lineage output of BM and FL transplanted HSCs into HA newborn mice.

In our study, FL cells, particularly FLE13 cells, were able to engraft and produce therapeutic levels of FVIII even in absence of preconditioning in newborn HA mice. This ability was not displayed by ABM cells, whereas both FL and ABM cells did not engraft in adults without preconditioning. Increasing circulating levels to 2-3% of normal FVIII activity can significantly reduce risks of spontaneous bleedings and

represent a clinically relevant achievement from the patient management point of view[43], even though several studies showed that levels of 20-30% may be required to prevent joint bleedings, while 3-5% can reduce to 1-2 joint bleeding episodes per year[4]. Despite the fact that our results were obtained using a preclinical mouse model of HA, we hypothesised that our strategy of cell transplantation without preconditioning could represent an alternative therapeutic approach which can improve patients' quality of life, while avoiding adverse effects of preconditioning chemotherapy. Additionally, despite the potential demonstrated in this study using a preclinical HA mouse model, FL cells will hardly be used in clinic for several reasons, such as availability, allogenic immune responses, therapeutic efficacy and ethical issues.

We are still far from fully understanding stem/progenitor cell engraftment in HA neonates, particularly related to conditioning regimens for efficient and safe endothelial progenitor cells engraftment. Studies related to engraftment of HSCs in wt mice are more advanced and alternative methods for HSC transplantation in adults have been described including transplantation of high number of HSCs in non-conditioned hosts[44] and novel conditioning methods inducing partial host BM ablation[45,46]. Moreover, it has been shown that active cell cycle enhances neonatal engraftment[31]. Considering that most of FL hematopoietic stem and progenitor cells are actively cycling from E11 to E13-13.5 whereas ABM cells divide infrequently[47] this could confer FL cells engraftment advantages under no-conditioning regimen.

We speculate that the difference in engraftment ability between FLE11 and FLE13 could be explained by HSC ontogeny and their commitment where FLE10.5 HSC migrate from aorta-gonads-mesonephros region (AGM) to FL, whereas FLE13 HSC start to move towards BM[31]. Additionally, previous transplantation studies using irradiated NOD/SCID mice have shown that the number and self-renewal activity of human lympho-myeloid stem cells within the CD34+CD38- population were similar in FL and cord blood (CB) and decrease during ontogeny in adult BM. However, although FL cells presented more self-renewal capacity (of approximately 7-fold to CB and 300-fold to ABM)[48] and high engraftment in an immunodeficient mouse model[49], CB and ABM cells showed higher output of mature myeloid cells (CD45/71+CD15/66b+)[48]. Also, CB cells can be an effective source of endothelial colony-forming cells (ECFCs), a subset of circulating endothelial progenitor cells, with recombinant FVIII production capacity and long-term endothelial engraftment

potential in newborn and adult HA mice[50]. Therefore, CB cells constitute promising fetal-like candidates for use as cell-based therapy for efficient treatment of newborn HA individuals. More studies are necessary to determine the output of human CB cells starting from preclinical models such as transplantation into immunodeficient newborn HA mice.

Further studies on different cell sources, in combination with the use of alternative non- or less-damaging conditioning regimens in newborns will lay the foundation to delineate molecular mechanisms involved in transplanted cells engraftment that should lead to new possibilities for bleeding phenotype correction in HA pediatric individuals. Moreover, we envisage the incorporation of gene therapy approaches, thus potentially increasing further FVIII production from transplanted cells.

Overall, this study has increased our knowledge on healthy FL cells and their possible usage in cell therapy approaches for the treatment of newborn patients. Future studies aimed at determining whether FL-derived hematopoietic and/or endothelial precursors would support higher engraftment ability and therapeutic potential compared to total FL cells could be envisaged. This study provides useful information regarding the hemato/vascular compartment reconstitution/repair in a neonatal preclinical model of HA, thus paving the way for studies focused at obtaining long-term reconstituting progenitors from other sources, such as CB or induced pluripotent stem cells (iPSCs), for the treatment of HA.

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FIGURES AND TABLES

Table 1. PB engraftment and FVIII activity in plasma of different groups of transplanted mice

Recipient mice	Donor cells	No. trasnplanted cells (x10^6)	No. positive mice / No. analyzed mice									
			4m						12m			
			no BU		BU2X		BU1X		no BU		BU2X	
			GFP eng.	FVIII act.	GFP eng.	FVIII act.	GFP eng.	FVIII act.	GFP eng.	FVIII act.	GFP eng.	FVIII act.
Newborn	FLE11	0,3-0,4			11/11	11/11	2/8	0/8			9/9	9/9
	FLE13	2-5	19/20	18/20	20/20	20/20	7/7	7/7	6/7	4/7	20/20	20/20
	BM	2-5	0/9	0/9	7/7	7/7						
	Control	0	0/6	0/6	0/21	0/21			0/6	0/6	0/21	0/21
Adult	FLE13	3-5	0/5	0/5	6/6	6/6						
	BM	5	0/5	0/5	5/5	5/5						
	Control	0	0/5	0/5	0/5	0/5						

GFP eng.= mice with GFP cells in PB ≥4%; FVIII act.= mice with FVIII activity ≥2%; control = not transplanted HA mice

Figure 1. Engraftment of FL cells into newborn HA mice. A) Schematic representation of the experimental procedure. HA pregnant mice received BU 2d and 1d prior to birth. At 2d after birth, HA mice were transplanted with FL E11-E13 cells from GFP+ mice. B) Engraftment of transplanted GFP+ cells, was evaluated by flow cytometry on peripheral blood cells at different time points up to 16 months after transplantation. GFP+ cells were detectable in peripheral blood of all transplanted mice (n=10-11). nº cells transplanted per recipient: FL11 $0.3\pm0.1x10^6$; FLE13 $5x10^6$. C) FVIII activity in plasma of transplanted mice was evaluated by aPTT assay. Murine FVIII activity was detectable up to 16 months in all transplanted mice. D) ELISA showing that none of transplanted mice developed anti-FVIII antibodies. Plasma samples were tested 12-16 months after transplantation (1:200 and 1:2000 = plasma dilution; Ctr+ = plasma from mice immunized with FVIII). E) Tail clip assay performed at 16 months after transplantation showed correction in all recipient mice (n=5 each group). Graphs are showing single values and mean values ± SD. Ctr+ = GFP+ mice (n=3). * $p<0.05$; ** $p<0.01$.

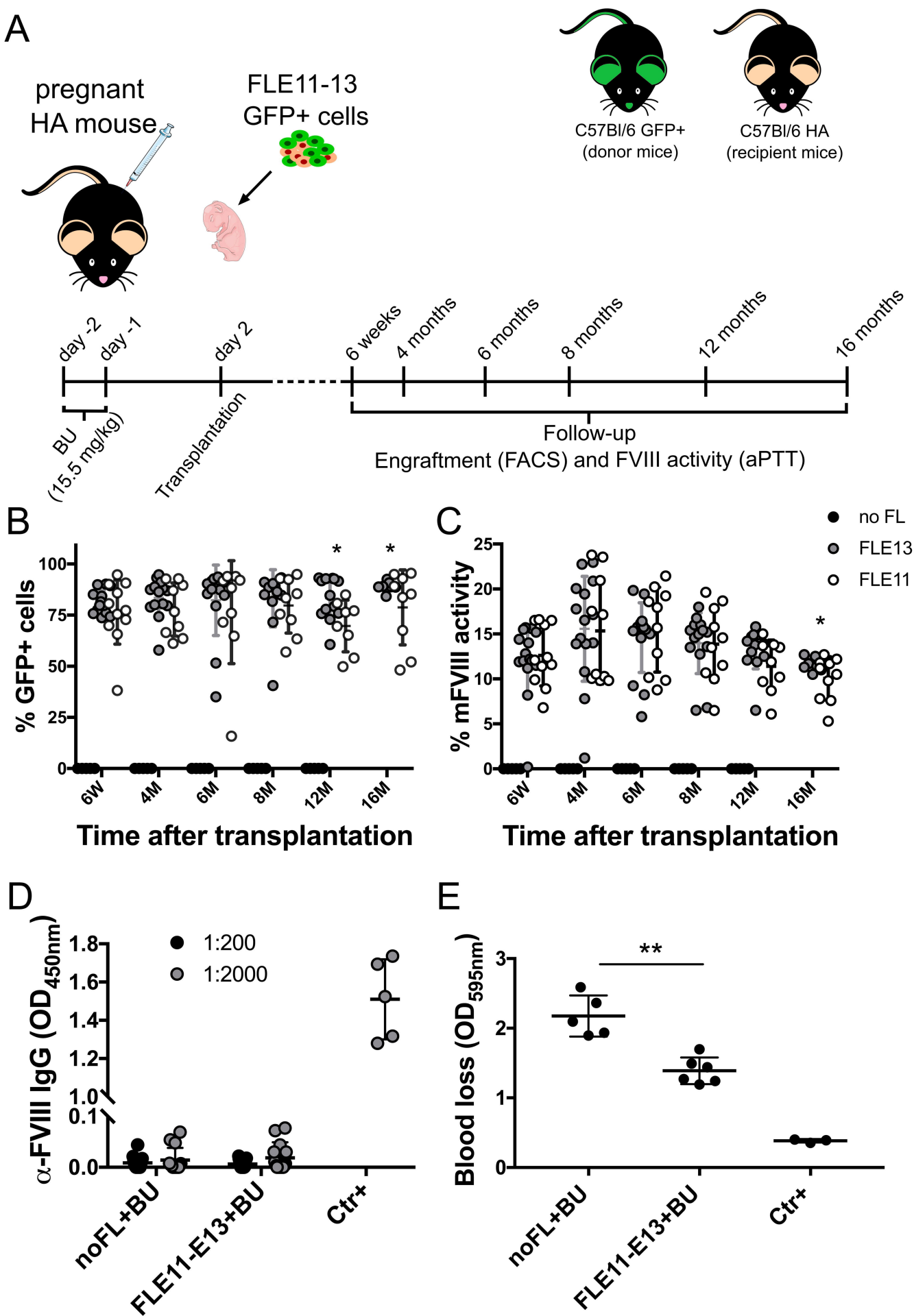
Figure 2. Engrafted donor cells characterization. (A&B) Representative flow cytometry analysis for characterization of GFP+ cells in spleen, bone marrow (A) and liver non parenchymal cell (NPC) fraction (B) 16 months after transplantation. C) Characterization of GFP+ cells in hepatic NPC fraction. Virtually all GFP+ cells in spleen, bone marrow and liver showed CD45 expression (A-C), while few GFP+ events (<1%) showed CD45- and CD31+ expression in NPC (B&C). (D&E) Flow cytometry data were confirmed by immunofluorescence in liver (D) and spleen (E) showing that GFP+ cells were virtually all CD45+. (F) mRNA expression analysis showed murine FVIII mRNA expression mainly in hemopoietic organs. Graphs are showing single values and mean values ± SD.

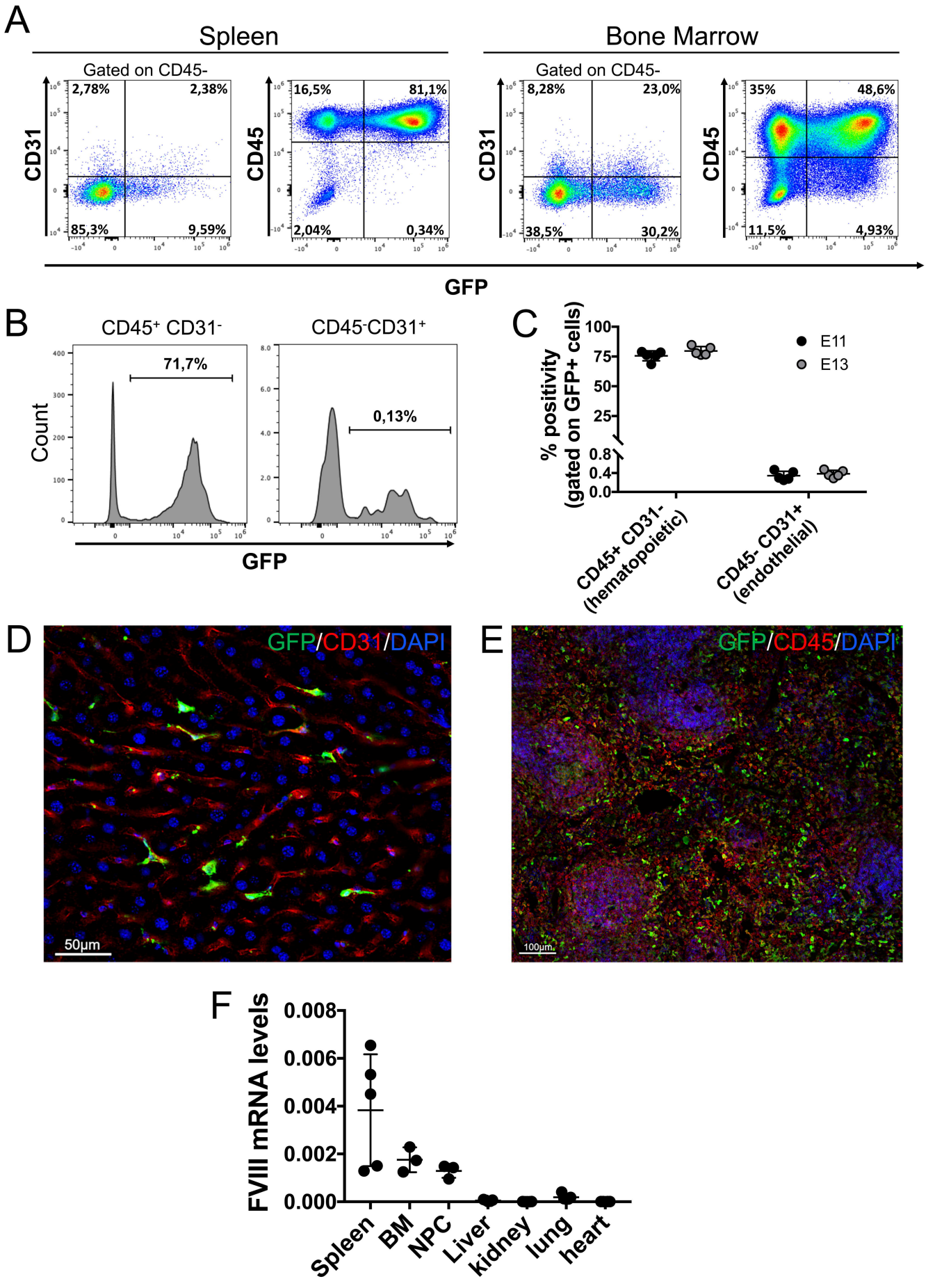
Figure 3. Effect of busulfan dosage on FL cell engraftment in newborn HA mice. 6 months following transplantation of FLE11 ($0.3x10^6$ cells per mouse) or FLE13 ($5x10^6$ cells per mouse) engraftment was ≥70% in mice treated with 15.5 mg/kg BU 48 and 24 hours before transplantation (BU2x). In mice treated with half BU dosage (BU1x) (15.5 mg/kg 24 hours before transplantation) engraftment significantly dropped but showed to be higher in mice receiving FLE13 compared to FLE11 (FLE11+BU2x, n=11; FLE11+BU1x, n=8; FLE13+BU2x, n=18; FLE13+BU1x,

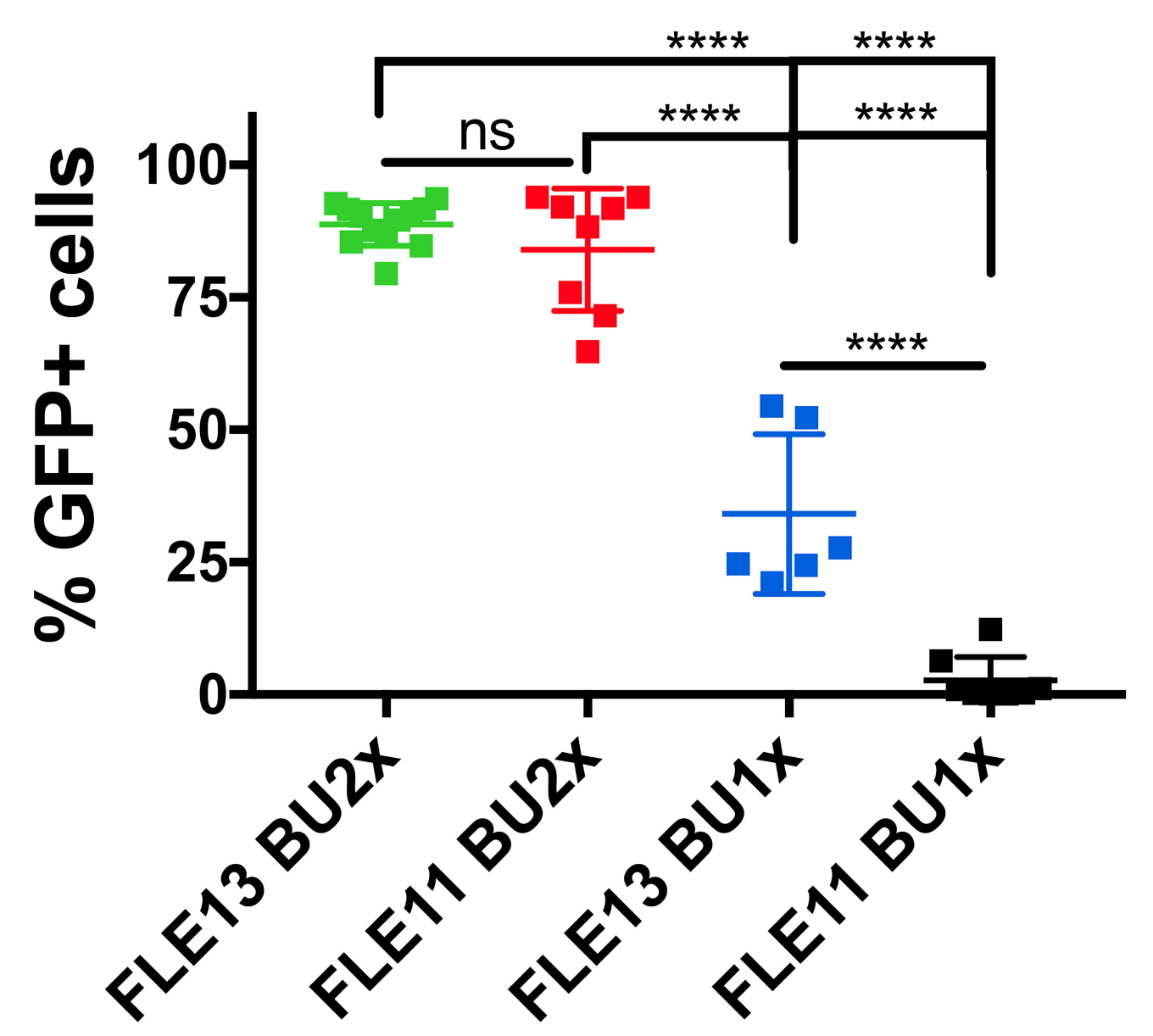
n=7). Graph is showing single values and mean values ± SD. ns= not significant, **** $p < 0.0001$.

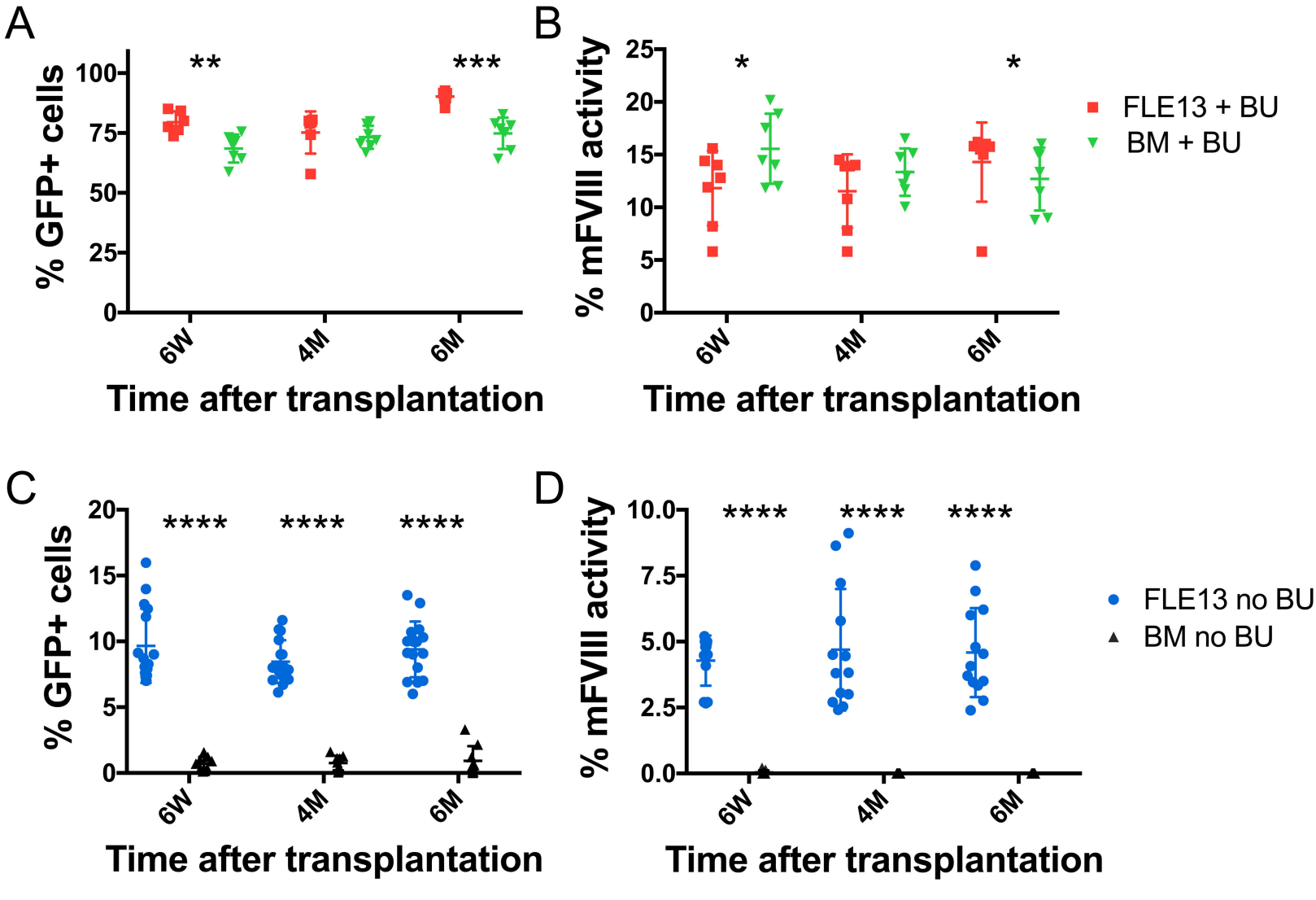
Figure 4. BM cell engraftment in newborn HA mice. A) Following transplantation in BU conditioned newborn HA mice, E13 cells engraftment was significantly higher than adult BM cells. B) Interestingly, mFVIII levels were higher in mice receiving BM cells, despite the lower engraftment level. (C&D) In mice receiving cells without prior treatment (noBU), FLE13 cells showed higher GFP+ cells engraftment (C) and mFVIII activity (D) compared to BM cells (n=8-10; *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Graphs are showing single values and mean values ± SD.

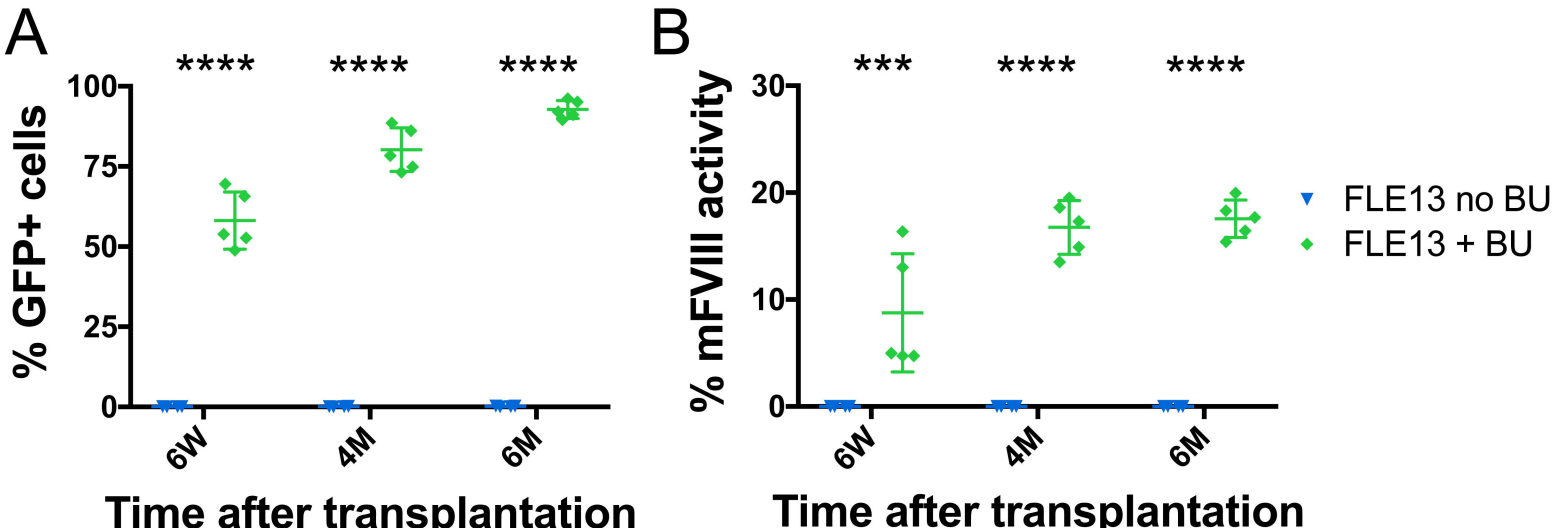
Figure 5. FL cells engraftment and bleeding correction in adult HA mice. FLE13 cells were able to engraft (A) and produce mFVIII (B) following transplantation in adult HA mice pre-treated with 2x30 mg/kg busulfan (BU), while no cell engrafted following transplantation in untreated adult HA mice (no BU) (n=5-6; *** $p<0.001$, **** $p<0.0001$). Graphs are showing single values and mean values ± SD.

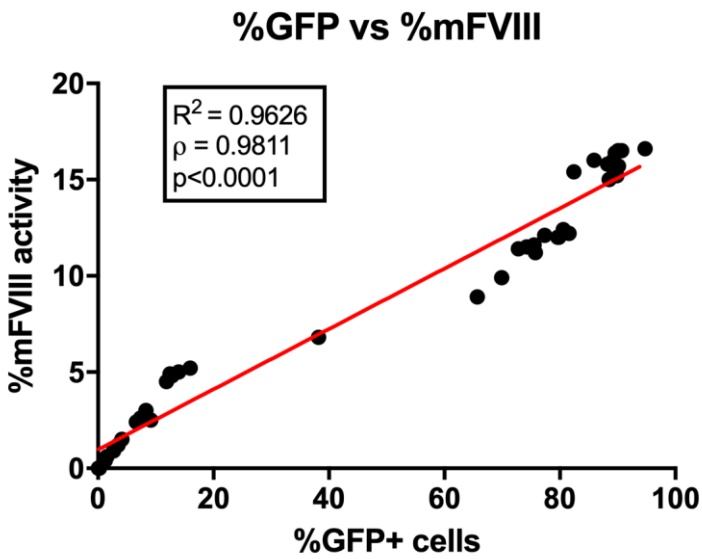

A
pregnant
HA mouse
FLE11-13
GFP+ cells
C57Bl/6 GFP+
(donor mice)
C57Bl/6 HA
(recipient mice)
day -2
day -1
day 2
6 weeks
4 months
6 months
8 months
12 months
16 months
BU
(15.5 mg/kg)
Transplantation
Follow-up
Engraftment (FACS) and FVIII activity (aPTT)
B
% GFP+ cells
Time after transplantation
6W
4M
6M
8M
12M
16M
C
no FL
FLE13
FLE11
% mFVIII activity
Time after transplantation
D
1:200
1:2000
α-FVIII IgG (OD450nm)
noFL+BU
FLE11-E13+BU
Ctr+
E
Blood loss (OD595nm)
noFL+BU
FLE11-E13+BU
Ctr+


A
Spleen
Bone Marrow
Gated on CD45-
Gated on CD45-
CD31
CD45
CD31
CD45
2,78%
2,38%
85,3%
9,59%
16,5%
81,1%
2,04%
0,34%
8,28%
23,0%
38,5%
30,2%
35%
48,6%
11,5%
4,93%
GFP
B
CD45+ CD31-
CD45-CD31+
71,7%
0,13%
Count
GFP
C
% positivity (gated on GFP+ cells)
E11
E13
CD45+ CD31- (hematopoietic)
CD45- CD31+ (endothelial)
D
GFP/CD31/DAPI
50µm
E
GFP/CD45/DAPI
100µm
F
FVIII mRNA levels
0.008
0.006
0.004
0.002
0.000
Spleen
BM
NPC
Liver
kidney
lung
heart

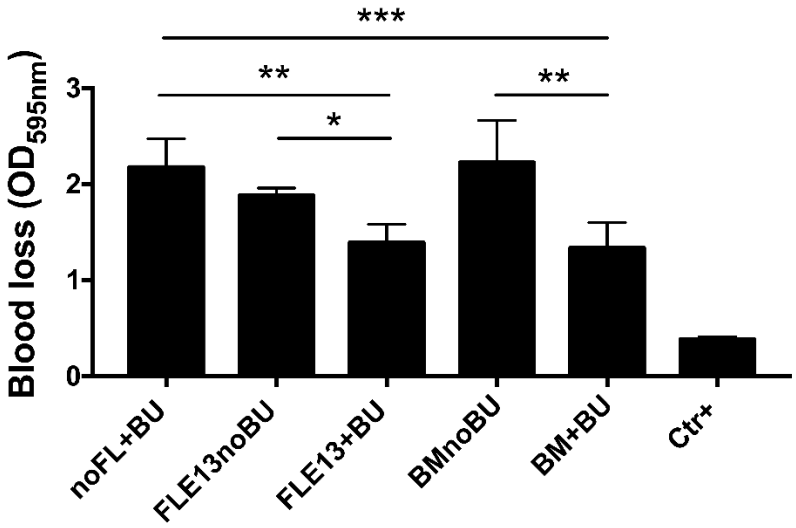

****
****
****
****
ns
****
100
75
50
25
0
% GFP+ cells
FLE13 BU2x
FLE11 BU2x
FLE13 BU1x
FLE11 BU1x


A
% GFP+ cells
100
75
50
25
0
**
***
6W
4M
6M
Time after transplantation
B
% mFVIII activity
25
20
15
10
5
0
*
*
6W
4M
6M
Time after transplantation
FLE13 + BU
BM + BU
C
% GFP+ cells
20
15
10
5
0
****
****
****
6W
4M
6M
Time after transplantation
D
% mFVIII activity
10.0
7.5
5.0
2.5
0.0
****
****
****
6W
4M
6M
Time after transplantation
FLE13 no BU
BM no BU

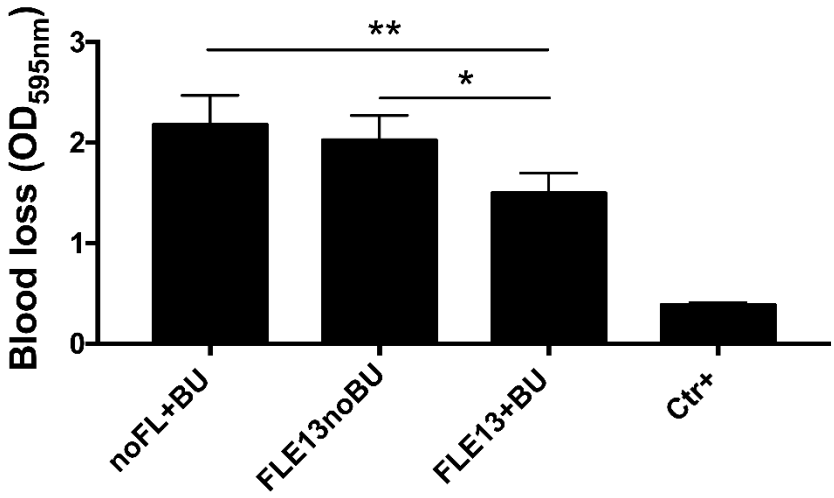

A
****
****
****
100
75
50
25
0
% GFP+ cells
6W
4M
6M
Time after transplantation
B
***
****
****
30
20
10
0
% mFVIII activity
6W
4M
6M
Time after transplantation
FLE13 no BU
FLE13 + BU



Supplementary Figure 1. GFP+ cells engraftment and mFVIII production correlation in transplanted mice. Pearson's correlation showing mFVIII levels correlating with percentage of engrafted GFP+ cells in a significant manner ($p<0.0001$) at 6 months post-transplant in HA mice with or without busulfan preconditioning.



Supplementary Figure 2. Bleeding assay in newborn HA following transplantation. Tail clip assay performed 6 months after transplantation showed correction in recipient mice pre-treated with BU and a lower correction only in not pre-treated mice receiving FLE13 cells. n=8-10 each group. Graphs are showing mean values ± SD. Ctr+ = GFP+ mice (n=3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.



Supplementary Figure 3. Bleeding assay in adult HA following transplantation. Tail clip assay performed 6 months after transplantation showed correction only in recipient mice pre-treated with BU (n=5), while mice receiving cells without BU pre-treatment did not show correction (n=4). Graphs are showing mean values ± SD. Ctr+ = GFP+ mice (n=3). * $p<0.05$; ** $p<0.01$.

Supplementary table 1. List of antibodies used for immunofluorescence and flow cytometry

Primary antibodies			
Antibody	Clone	Brand	Dilution
Rat anti-mouse CD45	IBL-5/25	Immunotools	1:200
Rat anti-mouse CD31	MEC 13.3	BD Biosciences	1:100
Rat anti-mouse F4/80	Cl:A3-1	AbD serotec	1:400
Rat anti-mouse Lyve-1	ALY7	eBiosciences	1:200
Rabbit anti-GFP	polyclonal	Molecular Probes	1:500
Secondary antibodies			
Antibody	Clone	Brand	Dilution
Goat anti-rat IgG	polyclonal	Molecular Probes	1:500
Goat anti-rabbit IgG	polyclonal	Molecular Probes	1:500
Flow cytometry antibodies			
Antibody	Clone	Brand	Dilution
Rat anti-mouse CD45	30-F11	eBiosciences	1:100
Rat anti-mouse CD31	390	eBiosciences	1:100
Rat anti-mouse CD309	Avas12a1	eBiosciences	1:50
Rat anti-mouse CD146	P1H12	eBiosciences	1:50

Supplementary table 2. Engraftment (% GFP+ cells) and correction (% mFVIII activity) levels in plasma of newborn mice according to BU dosage and cell transplantation.

Group	Treatment	6 weeks		4 months		6 months	
		% GFP	% FVIII	% GFP	% FVIII	% GFP	% FVIII
no FL	no BU (n = 7)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	BU (n = 10)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
FLE13	no BU (n = 12)	6.3 ± 1.7	2.7 ± 1.9	7.2 ± 2.2	2.4 ± 1.2	7.5 ± 4.5	2.3 ± 1.7
	BU 1x (n = 7)	30.9 ± 14.4	4.6 ± 1.2	38.9 ± 16.7	6.7 ± 2.7	30.4 ± 16.9	7.7 ± 3.7
	BU 2x (n = 12)	79.5 ± 4.5	12.8 ± 2.6	75.2 ± 8.8	12.5 ± 2.7	90.2 ± 2.6	15.7 ± 0.4

List of abbreviations

Abbreviation	Definition
HA	Hemophilia A
FVIII	Factor VIII
LSEC	Liver sinusoidal endothelial cells
HSC	Hematopoietic stem cells
BU	Busulfan
FL	Fetal liver
BM	Bone marrow
AAV	Adeno associated virus
MCT	Monocrotaline
GFP	Green fluorescent protein
aPTT	Activated partial thromboplastin time
NPC	Non parenchymal cells
qRT-PCR	Quantitative real-time polymerase chain reaction
PB	Peripheral blood
AGM	Aorta-gonads-mesonephros region
CB	Cord blood
ECFCs	Endothelial colony forming cells
iPSCs	Induced pluripotent stem cells