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PhD Thesis

Cell and gene therapy approaches to cure hemophilia A

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Abbreviations

aPTT	Activated partial thromboplastin time
BDD-FVIII	B domain deleted FVIII
BEC	Bladder epithelial cells
BM	Bone marrow
CB	Cord blood
FIX	Coagulation factor IX
FVIII	Coagulation factor VIII
HA	Hemophilia A
HSC	Hematopoietic stem cells
IF	Immunofluorescence
KC	Kuppfer cells
LpF8	Long FVIII promoter
LSEC	Liver sinusoidal endothelial cells
LV	Lentiviral vectors
MK	Megakaryocytes
NSG-HA	NOD-SCID-γNull hemophilia A mice
pF8	FVIII promoter
rFVIII	ricombinant FVIII
SpF8	Short FVIII promoter
TF	Transcriptional factor
vWF	von Willebrand factor

SUMMARY. Identification of cells capable of synthesizing and releasing factor VIII (FVIII) is critical for developing therapeutic approaches in hemophilia A (HA). Endothelial cells (EC), particularly liver sinusoidal EC (LSEC), express FVIII most in the body. In liver context we confirmed that human LSEC represent the major source of the protein, nevertheless, FVIII positivity was also detected in hepatocytes and Kuppfer cells (KC) at mRNA and protein level. Moreover, recent studies of bone marrow (BM) transplantation suggested additional cell types could synthesize and release FVIII, correcting the bleeding phenotype in HA mice. Therefore, to establish the ability of hematopoietic cells (HCs) in expressing FVIII, we analyzed several murine and human HC types. We found by RT-PCR and immunofluorescence (IF) that FVIII was present mainly in myeloid cells such as monocytes, macrophages, dendritic cells and megakaryocytes (MK) isolated from peripheral blood, mouse BM and human cord blood (hCB). These results were also confirmed by FVIII expression data coming from transcriptome analysis of several human cancer cell lines. Finally, we performed transplantation studies in immunodeficient NOD-SCID-γNull-HA (NSG-HA) mice (n=12) with CD34+ from hCB. FACS analysis showed engraftment higher than 40% in most of the mice up to 3 months after transplantation. Activated partial thromboplastin time assay (aPTT) performed on treated mice plasma at 12 weeks showed FVIII activity levels between 2% and 5% of normal, sufficient to ameliorate the bleeding phenotype; indeed 75% of mice survived after tail clip assay. Similar results were obtained in short term experiments injecting hCD11b+ cells in NOD-SCID.HA mice. To enlarge our knowledge on FVIII transcriptional regulation we investigated FVIII promoter (pF8) activity both in vitro and in vivo. By performing in silico analysis of transcriptional factors (TF) consensus sequences on pF8 sequence, we predicted the presence of several myeloid-specific TF, in addition to hepatocytes- and endothelial-specific TF. To evaluate pF8 activity in cells and tissues, we inserted in a lentiviral transfer construct the human pF8 sequences (short, SpF8 1175bp; long, LpF8 2350bp) driving GFP or FVIII expression. In vitro lentiviral vector (LV) transduction showed GFP expression in several hematopoietic, hepatic and EC with low intensity in hepatic cells. Since no differences in GFP expression were appreciated between LpF8 and SpF8, we used the SpF8 for further studies. Thus, we injected LV.SpF8.GFP in C57BL/6 mice and evaluated GFP expression by FACS and IF analysis in several organs at several time-points. GFP expression was restricted to hepatic non-parenchymal cells, meanwhile hepatocytes were barely detected. In particular, by costaining of GFP and LSEC or KC-specific markers revealed that pF8 was predominantly active in LSEC. Instead, in hematopoietic organs, such as spleen and BM, GFP expression was virtually restricted to myeloid cells. Ex vivo lin- LV.pF8.GFP transduced cells transplantation in mice demonstrated sustained GFP expression in hematopoietic cells, mostly in myeloid population, in blood and tissues. Additionally, in spleen and liver of transplanted mice GFP expression was restricted to F4/80 positive macrophages. Finally, we injected LV.SpF8.hFVIII in the tail vein of HA mice. aPTT assay demonstrated FVIII activity between 5 and 10% of normal in treated mice up to 32 weeks without anti-FVIII antibodies formation. Our results demonstrate that pF8 is differentially active in cell-subpopulations of several organs contributing to identify FVIII producing cells.

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Introduction

Hemophilia A

Hemophilia A (HA) is a recessive X-linked bleeding disorder that occurs in 1:5000 male new births and is due to the lack or reduced activity of coagulation factor VIII (FVIII) (1). FVIII is a non-enzymatic cofactor part of the coagulation cascade. When activated by thrombin FVIII is able to bind the factor IX (FIX) in a complex that activates factor X (FX), which converges in the common pathway of the coagulation cascade converting fibrinogen to fibrin and clot formation (Figure 1).

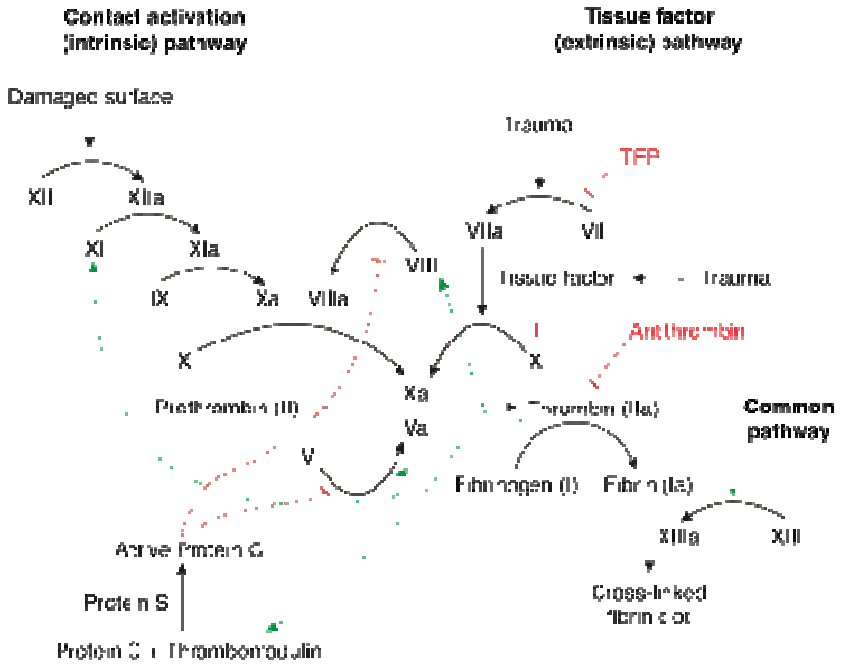


Figure 1. schematic representation of coagulation cascade

Based on the residual FVIII activity, there are three forms of hemophilia A: the severe form, in which the levels of FVIII are below 1%, the moderate form, between 1 and 5%, and the mild form, from 5 to 40% of FVIII activity (2). The most frequent mutation that affects approximately 45% of patients with severe hemophilia is the inversion of the first 22 exons caused by intrachromosomal recombination between the intron 22 (int22h1) with two homologous sequences that are distant 500kb from the gene (int22h3 and int22h3) (3). Other mutations causing the severe form are small deletions or insertions, missense and non-sense mutations and taken together they occur in 45% of patients. Less frequent mutations are large deletions, splice site mutations and intron 1 inversion (4). Instead, missense mutations are mainly associated with moderate and mild form (Figure 2).

Mutation Type	Mechanism	No. of Mutations	% of Mutations
Missense	Substitution	1091	50,9%
Nonsense	Substitution	252	11,8%
Frameshift	All	469	21,9%
	Deletion	339	15,8%
	Duplication	94	4,4%
	Insertion	19	0,9%
	Insertion/deletion	17	0,8%
Splice site change	All	154	7,2%
	Substitution	141	6,6%
	Deletion	12	0,6%
	Insertion/deletion	1	0,1%
Large Structural Change (>50 bp)	All	116	5,4%
	Deletion	99	4,6%
	Duplication	11	0,5%
	Insertion	4	0,2%
	Inversion	2	0,1%
Small Structural Change (in-frame, <50 bp)	All	46	2,1%
	Deletion	28	1,3%
	Duplication	3	0,1%
	Insertion	6	0,3%
	Insertion/deletion	9	0,4%
Synonymous	Substitution	11	0,5%
Promoter	Substitution	4	0,2%
Total		2142	

Figure 2. FVIII mutations frequency reported by CDC Hemophilia A Mutation Project (CHAMP)

Disease clinical manifestations range from spontaneous bleeding, with frequent haemarthroses in the most severe form, to secondary bleeding with rare haemarthroses in milder form (2). Diagnosis is made in relation to family history or following the first haemarthrosic episodes that occur at different ages according to the clinical form of hemophilia (0-3 years for severe, 2-7 for moderate, 5-14 for mild) (5). The main diagnostic technique used in laboratory is the evaluation of activated partial thromboplastin time (aPTT), which depends on the ability of patient's plasma to shorten the time required for clot formation in a standard FVIII-free plasma (6). However, this method can sometimes be inaccurate to diagnose the mild form and provides in 5-10% of cases false negative results. This implies the use of other more sensitive techniques such as two-stages clotting, chromogenic assay and genetic investigation (6). The current therapeutic practice consist

in the administration of recombinant FVIII or blood products and is carried out based on the disease severity; patients with severe hemophilia receive every other day prophylaxis, while patients with mild and moderate form receive replacement therapy on demand (7). Primary prophylaxis is defined as regular continuous treatment initiated in the absence of documented osteochondral joint disease, determined by physical examination and/or imaging studies, and started before the second clinically evident large joint bleed and age of 3 years. Continuous means the intention of treating for 52 weeks/year and at least 45 weeks/year (85%) (8). Patients receive 25-40 IU kg-1 per dose administered intravenously three times a week (8). The main complication related to this treatment is the development of neutralizing antibodies (inhibitors) to FVIII that occurs within the first 50 exposure days and is common in 20-40% of patients with the severe form. This worsens the clinical aspect because it makes ineffective the treatment and further reduces the residual activity of the endogenous FVIII (9). Among the risk factors for inhibitor formation the F8 genotype and the bleeding phenotype are highly predictive, indeed multidomain deletions are associated with nearly 90% risk to develop neutralizing antibodies (10). Another factor related to inhibitors development consist in FVIII haplotypes, indeed, for instance six protein variations were reported (H1-H6). H1 and H2 haplotypes are predominant in white population and are the most frequently associated with recombinant and plasma derived FVIII where as H3, H4 and H5 were reported only in black people (11). It was suggested that this haplotypes mismatch lead a two folds higher susceptibility to developed antibodies by African patients (11). Recent discovery showed that, in comparison with non-neutralizing antibodies, neutralizing antibodies mainly belong to the IgG4 subclass, display high affinity to FVIII and have long lifespan in circulation (12).

Cell mechanisms underlying the immune response to FVIII are not completely understood. However is likely that T helper lymphocytes (CD4+ cells) activated after FVIII presentation by antigen presenting cells (APC) plays an important role. Generally, the interaction between MHCII and T cell receptor triggers the first signal for CD4+ activation; even so, T cells become fully activated only in the presence of a costimulatory signal such as B7/CD28 or CD40/CD40L. This “two signal model” is required to generate T lymphocytes able to produce cytokines necessary to sustain B-cell differentiation, maturation and antibodies formation. An indirect evidence of CD4+-dependent inhibitory response was assessed in HA patients infected by HIV in the first years of replacement therapy. Indeed, in these patients the inhibitory titers decreased concurrently to the reduction of CD4+ cells and increased again after HIV therapy (13, 14). In addition, it was demonstrated that blocking costimulatory signal is sufficient to reduce antibody

titers in HA mice which have developed anti-FVIII inhibitors, proving that T cells are fundamental in the biological process ending with inhibitors formation (15, 16). Moreover, CD4+ cells isolated from patients who have developed anti-FVIII antibodies proliferated in vitro upon stimulation with exogenous FVIII (17, 18). The treatment of patients with inhibitors is particularly complicated and mainly consists in the administration of active FVII or activated prothrombin complex (bypassing agents) and in techniques to temporarily reduce the amount of immunoglobulin in the plasma (plasmapheresis and immunoadsorption) (9). However, these solutions are not as efficient as FVIII replacement to restore hemostasis and do not solved definitively the problem of inhibitors formation. For these reasons, the possibility to induce sustained FVIII tolerance represents the best alternative to overcome this issue. Immune tolerance induction (ITI) for patients who developed neutralizing antibodies consists in regular daily high dose administration of FVIII for several weeks, or even months, depending on protocol, aiming to eradicate inhibitors and to restore replacement therapy efficacy by inducing antigen-specific tolerance (19). The first ITI therapy was performed more than 30 years ago showing the ability of this treatment to eradicate antibodies in a 20 years old patient (20). In patients with high inhibitors titer (>5 Bethesda units) the success rate of ITI is between 60% and 80% with low relapsing of 15% in 15 years (21). Despite the great outcomes the high costs of ITI reduced its feasibility to few patients requiring the development of other strategies such as blockade of co-stimulation, oral tolerance, immunosuppressive treatment and antigen-specific regulatory T cell (22).

Replacement therapy

Before the advent of replacement therapy, life expectancy of patient affected from the severe form was less than 20 years, and the quality of life was generally devastating from joint bleeding complications or intracranial hemorrhage (23). Replacement therapy became feasible in the mid-1960s by discovering that precipitate left from thawing plasma was rich in FVIII (cryoprecipitate). Later, manufactories developed a methods to separate FVIII from pooled plasma, allowing to purify FVIII as lyophilized concentrate product (24). However, in the late 1970s the use of contaminating pooled plasma concentrated factors caused the widespread of serious infections such as HIV, HBV and HCV in hemophilia community (25). Subsequently, manufacturers introduced several viral inactivation treatments and the control of donors became more rigorous. Anyway great concern about the safety of plasma-derived products continued among hemophilia patients. The big advance in replacement therapy was achieved following the

FVIII gene cloning in 1984 which allowed the development of highly purified recombinant FVIII (rFVIII) (26). The first generation of rFVIII products were synthesized by gene-transfection in mammalian cells (Chinese Hamster Ovary cells) and were first used in clinic in the late 1980s (27). These rFVIII were produced in presence of animal or human derived protein in cell culture and contained human albumin in the final formulation as stabilizers (28). Safety was improved since 2000s replacing albumin with sucrose giving rise to the second generation of rFVIII (29). Among these new products generation was introduced in the market also a rFVIII lacking the B domain (BDD-FVIII) (ReFacto) that is dispensable for hemostasis but increase the efficacy in rFVIII production by cells (30). The third generation of rFVIII products are produced in absence of any animal or human proteins reaching the highest level of safety (31). Despite the development of blood products and recombinant FVIII has drastically improved the patients quality of life, replacement therapy do not represent yet a definitive cure and some issues are still to be solved. Among these, there are the high costs, the frequent number of administration due to the short FVIII half-life in circulation and the high probability to develop neutralizing antibodies. To extend rFVIII half-life several products were developed and most of them are now in Phase III clinical trial (24). Three general bioengineering mechanisms were proposed: (i) PEGylation to reduce receptor-mediated endocytosis, (ii) conjugation to the IgG Fc to avoid clearance by reticuloendothelial cell through the interaction with the neonatal Fc receptor (FcRn) and (iii) modification of rFVIII to enhance the binding with von Willebrand factor (vWF). (i) The majority of infused FVIII is cleared by the interaction with LDL receptor-related protein (LRP) that is abundantly expressed in the hepatocytes and KC other than in other organs (32). PEG can reduce this binding by incorporating a large number of water molecules and increasing the hydrophilic properties of FVIII (33). Progresses in FVIII bioengineering have allowed the possibility to conjugate in more specific way PEG to the target protein avoiding interference with other functional molecules, in the case of FVIII for example with vWF and tenase complex. Today three PEGylated rFVIII are currently in Phase III clinical trial, one of these is randomly conjugated (BAX 855, Baxter) and the other two are site directed PEGylated (BAY 94-9027, Bayer; NN7088, Novo Nordisk). All of them are reported to extend FVIII half-life by 1,5-1,7 fold compared with a commercial not modified rFVIII (34). (ii) Albumin and IgG share a very extent half-life of 19-22 days in comparison with the typical half-life of few minute or few days of other human plasma proteins (35). This is due to the interaction between these proteins with the FcRn. IgG entering in cells by pinocytosis are accumulated in early endosomes where bind the FcRn by a pH-dependent affinity due to acidity of this cell compartment (36). This interaction

avoids the catabolism of IgG and the complex is recycled to the cell membrane where the neutral pH of the bloodstream induces the dissociation of FcRn-IgG complex releasing the IgG back to the circulation (36). rFVIII-Fc developed by Biogen Idec/SOBI which has completed the Phase III clinical trial showed a 1,5-1,7 fold extension of FVIII half-life (37) (iii) Moreover, CSL Behring developed a single chain rFVIII in which the light and heavy chain are covalently linked demonstrating an higher binding affinity for vWF. For now this rFVIIISingleChain is in Phase I/II clinical trial (38).

Coagulation FVIII

FVIII gene (F8), which maps to band Zq28 at the tip of the long arm of the X chromosome was characterized firstly in 1984 (39). It measure 186 kb in length and is constituted by 26 exons encoding for a mature protein of 2332 amino acids (263 KDa) plus 19 aa signal oligopeptide at the N-terminal (40). FVIII molecule is organized in 6 domains: A1, A2, B forming the heavy chain and A3,C1,C2 that constitute the light chain. Between A1/A2, A2/B and B/A3 domains are present three acidic regions (a1, a2, a3) that contain the thrombin binding sites (Arg372, Arg740, Arg1689) and are crucial for FVIII activation (41). The overall structure of FVIII is similar to FV, in particular in the A domains they share approximately 40% amino acid identity also with the copper binding protein ceruloplasmin (42). Moreover C domain share some homology between FVIII, FV and proteins that bind negatively charged phospholipids (e.g., fat globular protein and the lipid-binding lectin discoidin I) (43, 44). Instead, FVIII B domain is unique and do not show significant similarity with FV or other proteins (45). Once synthesized, FVIII enters in the endoplasmic reticulum (ER) in which undergoes two important modifications: the elimination of the signal peptide and the introduction of oligosaccharide chains on asparagine residues predominantly arranged on the B domain. The N-glycosylation is fundamental to ensure the correct folding of the protein, to prevent the aggregation of intermediate forms and to allow the interaction of FVIII with enzymes and chaperon proteins essential to the correct intracellular processing, vesicular trafficking, exocytosis and secretion of FVIII (46). The ER-Golgi transition is mediated by the interaction between FVIII B domain with specific protein complex, in particular lectin-mannose binding 1 (LMAN1) also known as endoplasmatic reticulum-Golgi intermediate compartment 53 kDa protein (ERGIC53) and multiple coagulation factor deficiency 2 protein (MCFD2) (45). Defects in these molecules cause the combined deficiency of circulating FVIII and FV, which share in part the same intracellular processing of FVIII (47). In the Golgi, FVIII is cleaved close to the C-terminal region of B domain (after the aa 1313 and aa 1648)

producing an heterodimer consisting in the heavy chain (200 kDa) and the light chain (80kDa) that are not covalently linked by a divalent metal ion (mainly Cu2+) between the A1 and A3 domains. Finally, the processes that complete the intracellular maturation of FVIII are the modification of saccharide groups introduced in ER and the sulfurization of some tyrosines located in the acidic regions target of thrombin proteolytic activity (48).

In physiological conditions the FVIII concentration in plasma is 200-300 ng/ml and is associated with high affinity with the vWF, which is 50-folds in excess compared to the FVIII (49). The role of this interaction is to increase the half-life of FVIII by reducing the clearance and avoiding the inactivation by protein C. Moreover, the vWF prevents the premature association of FVIII with other coagulation factors before its activation mediated by thrombin. The regions involved in the binding between FVIII and vWF are located in correspondence of the acidic sequence a3, at the C-terminal of the B domain and in the C2 domain (50). Not surprisingly, therefore, those mutations in the gene of vWF or FVIII impairing the ability of interaction between the two proteins can produce very similar pathological phenotypes (50). In the early stages of the coagulation cascade, a little amount of activated thrombin acts on FVIII, and other coagulation factors, to amplify the hemostatic process. The FVIII amino acids involved in thrombin cleavage are Arg372, Arg740 and Arg1689, that are located in the acidic regions a1, a2, a3, respectively. After activation, FVIII dissociates from vWF and is able to bind platelets surface by hydrophobic interaction between some structural motifs of the C2 domain and the membrane phospholipids, which is further enhanced by the difference in charge between the two surfaces (51). In this conformation FVIII, is able to form a protein complex with FIXa and FX, known as tenase, which leading FX activation by proteolytic action of FIXa. The FVIIIa domains involved in the maintenance of tenase are A2 and A3 domains for the interaction with FIXa and the acidic region a1 for the binding with FX (52). Under physiological conditions, the clotting process triggered by any alteration of hemostasis must always end with the fibrin cap dissolution, to ensure a steady and regulated balance between a pro-thrombotic and anti-thrombotic phenotype. In this balancing thrombin plays a key role by activating coagulation cascade inhibition systems, including, the activation of protein C. Once activated this protease is able to inactivate several coagulation factors, among which, FVIII that is cleaved at the level of the A1 and A2 domains (52). In normal condition, FVIII half-life in circulation is relatively short (12-14h) even in the presence of vWF (53). This is due to the clearance mediated by several endocytic receptors such as LDL (low density lipoprotein) receptor-related protein (LRP), asialoglycoprotein receptor, VLDL receptor and the macrophage mannose receptor (54-57). Among these the more characterized and

effective in FVIII removal from circulation is the LRP which is a multiligand hepatic receptor belonging to LDL receptor superfamily. The interaction between FVIII and LRP1 was established firstly in 1999 by two separate groups (57, 58). Starting from this finding, it has been demonstrated that the blocking of LRP1 by infusion of an antagonist (RAP) results in FVIII clearance reduction with a longer half-life (57, 59). Additionally a conditioned mouse model in which LRP1 was specifically knocked out in the liver showed increase levels of FVIII (60). Several studies identified different FVIII domains involved in the binding with LRP. Initially, it was described that A2 domain amino acid region 484-509 and 558-565 displayed high affinity for LRP interaction (57, 61, 62). Furthermore, also peptide sequences in A3 and C1 domains are reported to be necessary for FVIII endocytosis (63-65). An important observation derived from the fact that alteration of such amino acids crucial for LRP binding did not affect the coagulation activity of FVIII (62, 63) providing a novel approach to improve FVIII half-life.

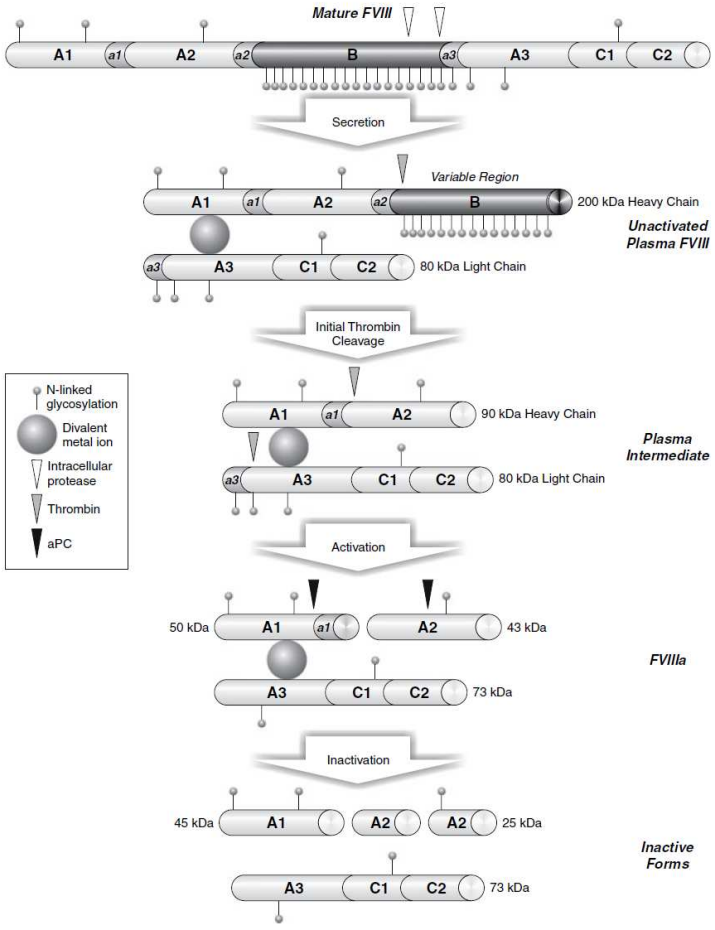


Figure 3. Schematic representation of FVIII protein during its lifecycle (45)

FVIII cell sources

Since orthotopic liver transplantation (OLT) corrected hemophilia A, liver has been considered the primary site of FVIII production (66-68). However, the identity of liver cells expressing FVIII was controversial and still now remains a question to be definitively clarified. Initially and for a long time hepatocytes were considered the FVIII expressing cells both at mRNA and protein level by *in vivo* and *in vitro* experiments (69-72). Although in early years the presence of FVIII was reported mainly in LSEC and macrophages rather than hepatocytes (73-75). These observation was strengthened by others (76) ending with the demonstration that LSEC but not hepatocytes secrete FVIII (77). Moreover, recent papers confirmed FVIII expression in endothelial cells (EC) by using a Cre/Lox strategy to selective knocking out FVIII expression in several cell types and concluding that FVIII is not synthetized in hepatocytes but mainly secreted by EC (78, 79). Furthermore, in addition to LSEC, several authors have reported FVIII transcript and protein also in EC of other organs such as kidneys, spleen and lungs (78, 80, 81) but not in heart and brain isolated EC (78). The importance of LSEC, both human and murine, in FVIII secretion was further confirmed by several transplantation studies in hemophilic mice (82-84). Indeed transplantation of healthy LSEC in HA mice restored therapeutic levels of FVIII activity and correct the bleeding phenotype of recipients up to 2 months (82). Conversely, the ability of hepatocytes to improve the clotting avtivity in HA mice after transplantation is not unequivocal and can depend by the site of transplantation. Ohashi and collegues demonstrated to achieve therapeutic FVIII levels up to 5 weeks by transplanting hepatocytes under the kidneys capsule of HA mice (85). Otherwise, intraperitoneal transplantation of hepatocytes do not provide detectable FVIII activity in recipients plasma in a short term experiment (1week) (84). The authors hypothesized that this result was due to the absence in the peritoneal cavity of vWF required to stabilize FVIII (84). Even if liver represent the main source of FVIII many studies suggested the presence of extrahepatic sites of FVIII production. The first evidence that other organs were able to express and secrete FVIII was reported in 1971 when Webster at al. demonstrated in dogs that transplantation of hemophilic liver in normal recipient caused only a partial reduction of circulating FVIII (86). More recently a similar result was obtained also in humans by Madeira (87). In this study a patient affected by stage 2 hepatocellular carcinoma with normal FVIII levels was transplanted with a liver coming from an hemophilic patient died for a diffuse brain injury. In the days after transplant, aPTT seconds of recipient did not increased meaning normal FVIII levels in circulation deriving from sources different from liver (87). Among the other organs, spleen transplantation showed to temporarily increase FVIII expression in HA dogs (88-90) and

more encouraging in humans, hemophilic patients transplanted with healthy spleen reached long term FVIII activity which ameliorate the clinical feature of the disease (91, 92). Other than spleen FVIII mRNA and protein were also detected in the glomeruli and in the tubular epithelial cells of kidneys and in mesenchymal cells isolated from different sites (93-96). Despite FVIII cDNA was firstly cloned from a T-cell line (97), the FVIII expression in hematopoietic cells was uncertain and poorly investigated. However, an early study described the presence of FVIII protein in pulmonary alveolar macrophages and in cells present in the splenic red pulp (75). A recent insight on a novel extrahepatic sources of FVIII demonstrated that BM transplantation significantly contribute to hemostasis in HA mice by rescue therapeutic levels of FVIII up to 6 months after treatment and allowing survival of mice after tail clip assay (93). This study highlights the role of hematopoietic cells in FVIII synthesis and secretion and offers a new potential therapeutic approach for hemophilia A. Despite these discoveries, the contribution of other sources of FVIII may be investigated and for instance little is known about FVIII transcriptional regulation. At this point the characterization of FVIII promoter activity could offer new insight to extend our knowledge about FVIII expression. Initially studies on F8 gene reported a 1175bp region upstream the ATG and containing the transcription starting site (-170) that probably was involved in transcriptional regulation of FVIII (39). Further investigation was later performed by Figueiredo at al. in 1995 by cloning the 1175bp proximal region in a luciferase plasmid system and testing the promoter activity in hepatic cell lines. By this strategy they identified a 200bp region containing the element for the maximal promoter activity and chromatin immune precipitation assay (ChIP) demonstrated the ability of 4 hepatic transcriptional factor (TF) such as HNF1, HNF4, C/EBPα and C/EBPβ to interact with FVIII promoter (98). However, this study did not analyzed the promoter activity in other cell types *in vitro* or *in vivo* and for instance the characterization of this region was not more deeply investigated.

Gene therapy

Gene therapy is a form of molecular medicine that has developed since the early nineties and is still evolving: the main purpose is to introduce into the target cells (but also in tissues or organs) DNA sequences in safe and efficient way, with the ultimate goal to obtain a therapeutic effect, or at least slow the disease progression. Fundamental point for gene therapy is to develop a system capable to achieve an efficient gene transfer in tissues without causing risks to the patient. For this reason, initial efforts have been spent to develop a system with these characteristics, in order

to transfer therapeutic gene in the cells directly into the body (*in vivo*) or in cells taken, modified, and finally reintroduced (*ex vivo*). Whatever the strategy used, gene transfer required the use of a vector able to overcome the biological barriers between cells and surrounding environment and capable to transfer the DNA into the cells. Vectors for gene therapy are commonly divided in viral and non-viral. Examples of non-viral gene delivery are naked/plasmid DNA, lipofection and nanoparticles however viral vectors are the major employed in gene therapy (Figure 4). These vectors derived from an extensive engineering of several classes of virus in order to satisfy the biosafety requirement and to make them able to carry the gene of interest. The most characterized and used viral vector in gene therapy application are those derived from adenovirus, adeno associated virus, retrovirus and lentivirus (Figure 4). The first successful gene therapy clinical trial in humans was achieved in the 1990 for the treatment of an inherited disease: the adenosine deaminase deficiency (ADA) who leads to a severe immunodeficiency. In this trial patients were infused with autologous T cell corrected *ex vivo* using a retroviral vector carrying the adenosine deaminase gene. As result, ADA was cured in one out two enrolled patients (99). Up to day, more than 1900 clinical trials were approved over the world for the treatment of several disease using different vectors. Although monogenic diseases would appear the ideal target for gene therapy, this approach was applied for many other disorder, first of all for cancer (Figure 5).

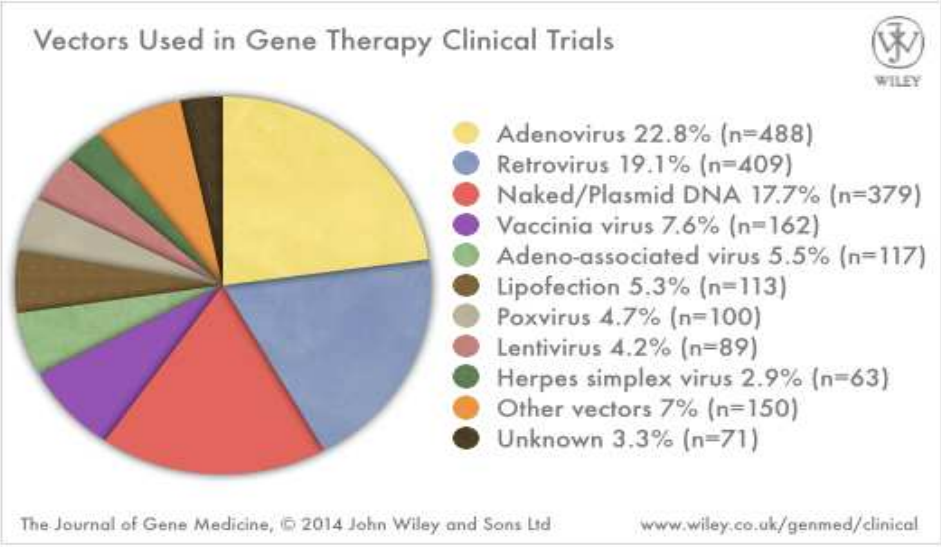


Figure 4. Diagram representing the vectors used in gene therapy

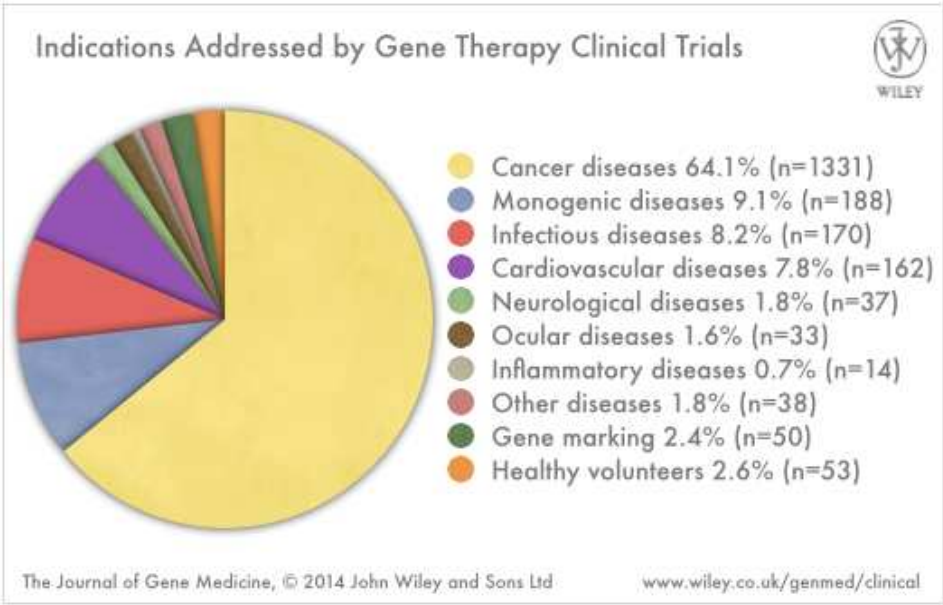


Figure 5. Diagram representing the diseases treated by gene therapy.

As shown by the initial success, combination of gene and cell therapy when applicable, provide a great solution for several diseases. Historically, BM transplantation represented one of the most practiced example of cell therapy for many applications such as oncohematology, anemias and immunodeficiencies. The first allogenic BM transplantation was performed in 1957 on six cancer patients under chemo and radio therapy regimen by E. Donnall Thomas (100) and to date it is estimated that in the world over than 50000 patients per year have been treated with BM transplantation for various diseases (101). However, for some pathologies, for example for monogenic disease where autologous transplantation is useless, allogenic solution is the unique possible approach, which poses several concerns. Among the most relevant there is the graft versus host disease (GvHD). This reaction occurs in the absence of full histocompatibility between donor and recipient in allogeneic transplants and corresponds to the cytotoxic action mediated by the transplanted cells, which once reaching the competence, attack the recipient organism (102). Gene therapy associated with BM or hematopoietic stem cell (HSC) transplantation combines the self-renewal properties of HSC providing a sustained expression of transgene with the possibility of autologous transplantation overcoming the GvHD. This strategy was used in different clinical trial over the years, mainly for the treatment of immunodeficiency (103, 104). Very recently, an italian group combines with success gene and cell therapy using lentiviral vectors (LV) in a clinical trial for two severe and lethal diseases: Metachromatic leukodystrophy and Wiskott-Aldrich syndrome (105, 106).

Lentiviral vectors

Lentiviruses are a class of single-strand RNA (ssRNA) based virus belonging to the retroviridae family, associated with slow and progressive disease characterized by immune deficiency and neurological disorders; among these viruses there are those that caused acquired immunodeficiency in humans, primates and other mammals (HIV-1 and 2, SIV, FIV, BIV and CAEV). The human immunodefiency virus (HIV) that caused the acquired immunodeficiency syndrome is the most characterized and employed for gene transfer purpose. Similarly to all retroviruses, lentivirus replicate through a DNA intermediate but unlike the onco-retroviruses are able to integrate their genome even in post-mitotic cells such as neurons and macrophages (107). The virus particle contains two identical copies of 9,5Kb long ssRNA. The genome is constituted by 9 genes arranged on more than one reading frame. In addition to genes encoding for the structural and enzymatic proteins (gag, pro, pol, and env), there are other genes for accessory (vpu, vpr, nef and vif) and regulatory proteins (tat and rev) that are involved in the expression, assembly and in the pathogenicity of the virus (108). The lentiviral vectors used for gene therapy exploiting only the first stage of the viral replicative cycle to transfer and integrate the transgene within the chromatin of target cell. Once integrated, vector proviral DNA is defective for some sequences required for genome production and for proteins necessary to assemble new virion particles in transduced cells (109). In order to build safe vectors the lentivirus genome was modified over the years by segregating the cis- and trans-acting sequence in different plasmid constructs. Cis-acting sequences that regulate the incapsidation (ψ sequence), the reverse transcription (PBS, cPPT-CTS e 3'PPT and R), the nuclear translocation (cPPT-CTS) and the integration of provirus genome (the att sequence present in the U3 and U5) were maintained in the transfer construct in which is inserted the expression cassette. Another cis-sequence preserved in the plasmid design is RRE, the Rev responsive element important for RNA transport to the cytoplasm (Figure 6). To improve safety, self-inactivating transfer constructs were developed by eliminating part of U3 enhancer/promoter from parental LTR (ΔU3) that was replaced by CMV enhancer/promoter retaining only a short U3 sequences required for genome integration (110). This strategy avoid the synthesis of genome RNA in transduce cells reducing the risk to produce replication-competent virus. Further, truncated LTR limit the interference with the expression cassette internal promoter and reduce the de-regulation of gene harboring the site of integration (110) (Figure 6). Other elements were then included in the transfer construct to improve transduction efficacy of LV. One of these is the central polypurine tract (cPPT) that facilitates

nuclear import of the lentiviral preintegration complex (PIC) before vector integration (111, 112). In addition the woodchuck hepatitis posttranscriptional regulatory element (WPRE) inserted after the transgene increased mRNA export, polyadenilation, and half-life (113) (Figure 6).

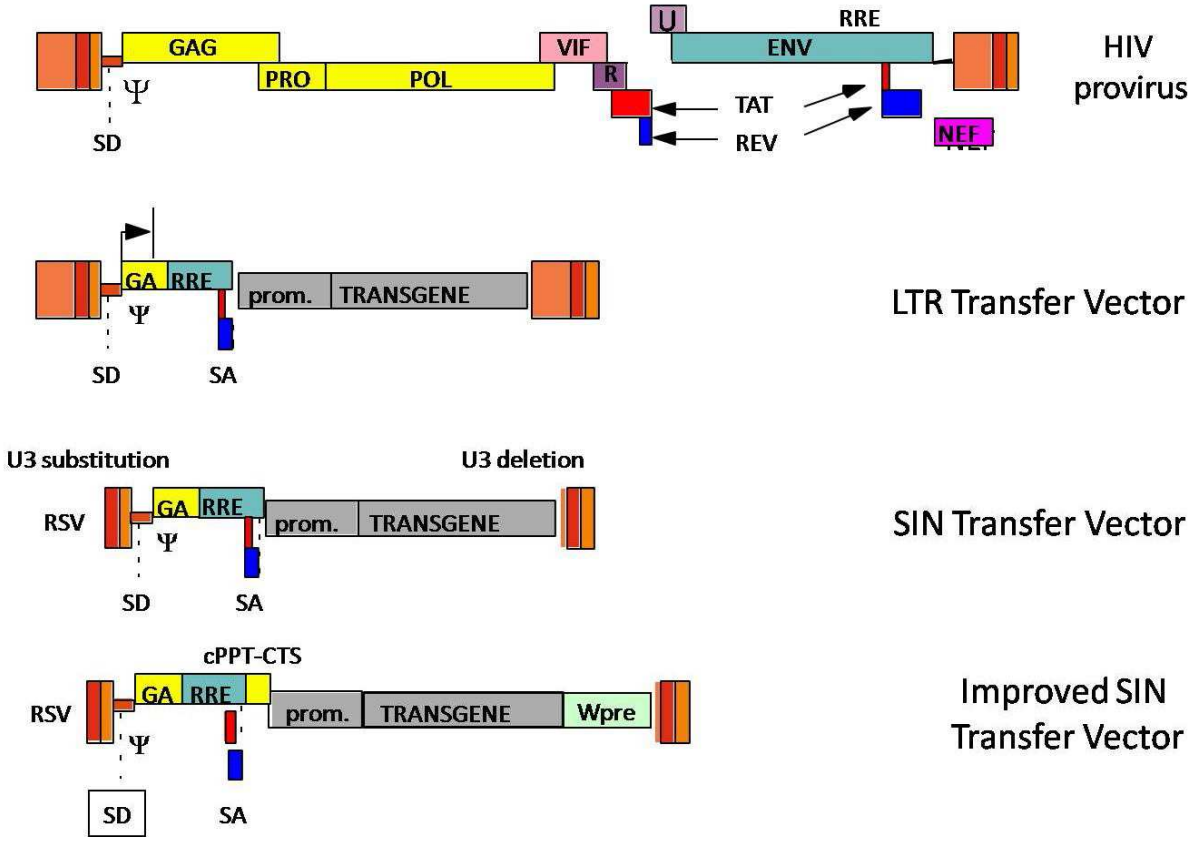


Figure 6. Schematic representation of modification made on LV transfer construct over the years

To improve vector biosafety extensive modifications were realized also on packaging construct. In the first generation of LV packaging construct part of the ENV was partially eliminated, but RRE sequence was maintained, LTRs were removed and the packaging sequence of ψ was mutated. LTRs were removed and replaced with the stronger promoter of cytomegalovirus (CMV) and polyA of chicken beta actin was inserted to substitute the parental polyA (114). Further, LV was pseudotyped by replacing HIV-1 envelope gene with the vesicular stomatitis virus glycoprotein (VSV-G) gene, increasing the cellular tropism virtually to all mammalian cells and augmenting the particles stability (114) (Figure 7). In this generation, packaging function was separated in two different construct. In the second generation of packaging construct the four accessory genes (Nef,Vip,Vpu,Vpr) were removed leaving gag and pol genes, which encoded for the structural and enzymatic components of the vector particles, and tat and rev genes with transcriptional and post-transcriptional functions (115) (Figure 7). In the third generation the

remaining genome was split in two plasmids: a packaging construct containing the gag and pol genes and a second plasmid containing rev gene and removal of tat gene (116) (Figure 7).

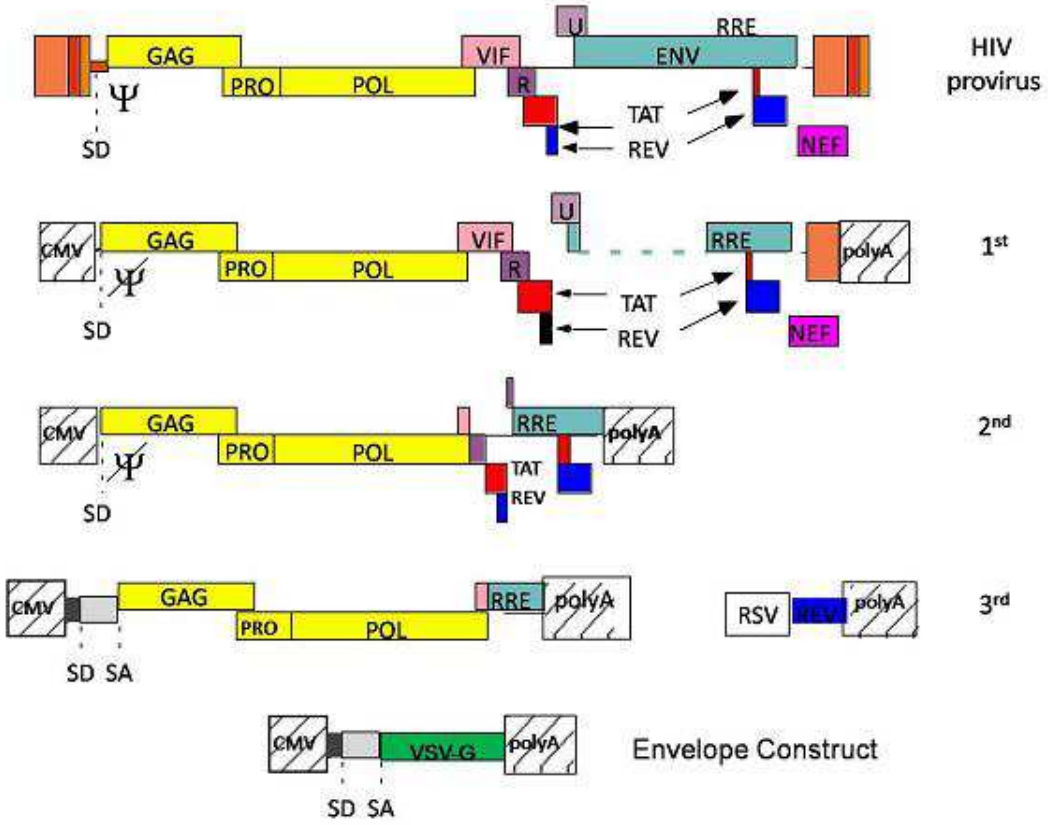


Figure 7. Schematic representation of modification made on LV packaging constructs over the years

Actually, the SIN transfer vector and the third generation packaging construct represent the best LV platform regarding biosafety and transduction efficacy. Since LV are integrating vectors the genotoxicity represent a big concern even because in an initial X-linked combined immunodeficiency (SCID-X1) ex vivo gene therapy clinical trial using a murine oncoretroviral vector carrying the interleukin 2 γ-chain some patients developed leukemia (117, 118). Further analysis showed vector integration close to LMO2 proto-oncogene promoter. Likely, retroviral LTR have induced LMO2 overexpression triggering unregulated cell proliferation and clonal expansion. This reflects the natural tendency of retrovirus to integrate their genome near the start of transcriptional unit (119). Conversely, LV integrate preferentially in active genes or in local hot spots but far from transcriptional regulation sequence (120). Indeed, under equal experimental conditions, in comparison with retroviral vectors, LV showed to promote low

insertional mutagenesis event and low tumors development (121, 122). Moreover, clinic outcome of several gene therapy trials using LV did not report any oncogenic occurrence (123-125).

Since regulation of transgene expression in define cell types could be important for some gene therapy applications several strategies were approached. First, is possible to modify the LV tropism by replacing the most common VSV-G envelope with others viral proteins which have an intrinsic tissue specificity or display more efficacy to transduce certain cell types. For example, baboon envelope pseudotyped LV are more efficient to transduce un-stimulated or resting CD34+ cells in comparison to VSV-G (126). The H and F envelope from paramixoviridae family instead provide to LV high targeting and rate of transduction to non-activate B and T lymphocytes (127). In addition, to restrict targeting selectively to hepatocytes the baculoviridae gp64 envelope was used (128). Regarding the central nervous system, great interest get the observation that lymphocytic choriomeningitis virus (LCMV) envelope showed to target specifically astrocytes, the main cells involved in glioma formation (129). Despite the targeting issue, VSV-G pseudotyping has associates with some drawback such as in vivo dose-dependent cytotoxicity and the susceptibility to be recognize by human serum complement (130, 131). A second level of targeting is obtained by working on transcriptional regulation. Ubiquitous promoters such as CMV, spleen focus forming virus (SFFV), human polypeptide chain factor-1alpha (EF-1alpha), phosphoglycerate kinase (PGK) allowed to a strong transgene expression, even though they are affected by some limitations. Among these there are the trend to be inactivated over the time impairing a sustained transgene expression and the presence of enhancer sequence within the promoter increased risk of insertional mutagenesis (132, 133). Furthermore, the ubiquitous transgene expression may be detrimental. This is the case of adverse reaction given by proteins ectopically expressed or citotoxicity mediated by suicides genes or toxin used for anti-tumor gene therapy (134-136). Another issue is represented by the innate or adaptive immune response against the transgene once is expressed by antigen presenting cells (APC) (137, 138). At this point the use of cell specific promoter to de-targeted transgene expression in APC allowed the sustained expression of therapeutic gene in the selected cells, for example hepatocytes, by reducing immune response (139-141). For these reasons, over the years many authors have used several tissue specific promoters to drive transgene expression in cells of interest including endothelial, hepatocytes, dendritic cells, hematopoietic stem cells, megakaryocytes, B cells etc (142-147). Finally, a more recent post-transcriptional regulation based on miRNA biology provided an additional degree of cell targeting. For gene transfer purpose, the insertion of complementary sequences to a specific miRNA (miRNA target sequence, mirT) to the 3' of the

expression cassette, offers the possibility to reduce selectively the transgene synthesis in the cell types in which that particular miRNA is expressed. This strategy was firstly developed by Brown and colleagues to avoid transgene expression in APC showing that the presence of miRT142-3p, complementary to miRNA 142-3p which is selectively expressed in hematopoietic cells, limits immune response against GFP by preventing transgene expression in those cells (148). Since miRNA is differentially expressed also during cell differentiation miRTs can be useful to regulate transgene expression in define stages of cell maturation. This characteristic was exploited by Gentner at al. to correct globoid cell leukodistrophy using the miRT-126 to de-target GALC expression which is highly toxic in the early stages of hematopoietic differentiation and allowing its synthesis in mature cells when miRNA-126 is downregulated (149).

Gene therapy for hemophilia A

Gene therapy could constitute a powerful therapeutic approach for many diseases, in particular for monogenic diseases. Additionally, hemophilia A represent an ideal target for gene therapy since restoring FVIII levels higher than 1% is sufficient to ameliorate the bleeding phenotypes of patients with an overall increase of quality of life. Hemophilia B gene therapy has provided good results in clinical trials by using adeno associated-viral vector (AAV) to deliver FIX into the patients. In a first clinical trial a sierotype 2 AAV vector carrying the FIX cDNA under the control of the ubiquitous cytomegalovirus promoter (CMV) was intramuscularly injected in 8 enrolled patients affecting by the severe form of hemophilia B (FIX activity less than 1%) (150). By this approach no local and systemic toxicity was reported and no immune response against the vector occurred even in case of pre-existing immunity (150). Despite the evidence of gene transfer and transgene expression, only modest circulating FIX levels (<1%) were achieved in most of the patients (150). The same group has repeated the study by injecting the AAV2 into the hepatic artery and changing the ubiquitous CMV promoter with an hepatocyte specific promoter (human alpha-1-antitrypsin) to drive FIX expression selectively to hepatocytes. However, gene therapy efficacy was hampered by a strong immune response against the vector with subsequent CD8 mediated destruction of transduced hepatocytes (151). Best results were obtained in a third clinical trial by using an AAV pseudotyped with AAV8 capsid protein. It has been reported that in comparison to AAV2 the AAV8 capsid has stronger liver tropism, lower seroprevalence in human and provide less virus uptake by antigen presenting cells. Moreover is able to mediate effective transduction in animals with pre-existing immunity to AAV2 (152). Regarding the expression cassette, an artificial liver specific promoter (LSP) was used to drive the expression of

a codon optimized FIX cDNA (153). Finally, the AAV8.LSP.FIX was administered by peripheral vein injection in 10 patients enrolled in the study. By these strategies FIX activity was restored at level between 2-6% up to a median of 3,2 years. However 4 patients were treated with prednisolone following the increase of liver enzyme and all of them have received at least one recombinant FIX infusion (154). Nevertheless, this clinical trial showed the feasibility of gene therapy for hemophilia B, increasing the quality of life of patients and encouraged new efforts to improve this approach making it a suitable alternative to replacement therapy. Despite the relevant results obtained for hemophilia B, gene therapy for hemophilia A has seen significantly less progress into the clinic due to some factors that complicates FVIII expression in comparison with FIX. (i) The size and complexity of FVIII (9 kb) make it too large for some vector system, such as AAV. (ii) Using a comparable vector delivery, transduced cells express 100 fold less FVIII level than FIX (155) (iii) FVIII is naturally 5-6 fold more immunogenic than FIX, making the transgene mediated immune response a big concern. For instance, several approaches for hemophilia A gene therapy using different vector systems were attempted.

AAV vectors are impaired by their limited capacity to packaged genome larger than 5 kb. To circumvent this problem FVIII light and heavy chain were split in two distinct AAV and upon co-injection in mice, biologically active FVIII was detected in circulation (156, 157). However, since the interaction between the two chains occurs inside the cells, it is necessary that both vectors co-transduce the same cell to allow the production of functional FVIII. This quite reduced the efficiency of the strategy and concurrently increased the overall dose of vector to use. Another option to overcome the size limit of AAV vectors was to use a B domain deleted FVIII that reduced by one third the final size of cDNA without compromising the biological activity of the protein. Even though a minimum promoter is required to not exceed the package capacity of AAV. By this attempt, two studies have showed sustained FVIII levels in mice (158, 159). However, in both, the authors have reported in most of treated mice anti-FVIII antibodies formation that was overcome by the second group by using the sierotype AAV8 instead the AAV1 (158). AAV vectors containing the BDD-FVIII have demonstrated to be suitable to induce FVIII expression also in hemophilic dogs (160, 161). Even so the doses needed to reach therapeutic correction were significantly higher than the maximum doses of AAV-FIX administered to human in clinical trials (162) and given the dose-dependence of immune response to capsid, the use of this vector could be not feasible in humans. The reduction of doses could be obtained by increasing FVIII expression, and it was gained by using a full length promoter (163). Although this required an oversized AAV genome (5-7,5 kb) that has reported to reduced

packaging efficiency (164). Additionally, a recent study has demonstrated to induce remarkable therapeutic expression of FVIII in non-human primate by targeting FVIII expression in hepatocytes combined with the use of a codon optimized FVIII (165). However, most of macaques needed transient immunosuppression to reduce anti-FVIII antibodies titer (165).

Among the other viral vectors lentiviral and retroviral were the more employed for the treatment of hemophilia A. The first proof of concept for *in vivo* gene therapy using a γ-retroviral vector was assessed in neonatal hemophilic mice. Approximately 50% of injected mice expressed physiological or even higher FVIII levels that were sustained up to 14 months. In this study, the remaining animals that showed only transient or undetectable FVIII expression developed anti-FVIII specific antibodies (166). This approach was also successful in a canine model of hemophilia A by targeting transgene expression in the liver without antibodies formation (167). However when retrovirus was used for human gene therapy only low circulating FVIII was detected in patients (168, 169). In 2000, Park and colleagues demonstrated that also LV could be used *in vivo* to induce human FVIII expression in wild type mice. By intraportal injection they direct FVIII expression predominantly to the liver with an ubiquitous promoter (EF1alpha) and reaching human FVIII levels of about 15%. Unfortunately hFVIII expression was only transient due to antibodies formation despite the fact that mice were not hemophilic and normally expressed murine FVIII (170). Later, similar results (5% of activity) was obtained in hemophilia A mice by intraperitoneal (IP) injection or *ex vivo* bone marrow transduction using a LV carrying the BDD-FVIII under the control of an ubiquitous promoter. However also in this case neutralizing antibodies were developed, with higher frequency in IP group rather than in ex vivo bone marrow transduction (171). Best outcome was achieved by improved targeting FVIII expression to hepatocytes. To do this a feline immunodeficiency virus-(FIV) based LV was pseudotyped with the baculovirus GP64 envelope that has been demonstrated to have a strong tropism for hepatocytes. Additionally, to further restrict transgene expression to these cells an hybrid hepatocyte-specific promoter (murine albumin enhancer/human alpha1-antitrypsin promoter) was included in the expression cassette. Liver targeting demonstrated to allow long-term therapeutic levels of FVIII without antibodies formation. Although slight inhibitors titer occurred by changing strain of mice, from C57BL/6 to a more immune reactive BALB/c background (128).

Up to day liver and in particular hepatocytes have represent the favorite target for hemophilia A gene therapy (165, 166, 170, 172-176) by their behavior to limiting transgene mediated immune response. Nevertheless, the anti-FVIII antibodies development is still a current drawback for the

feasibility of hemophilia A gene therapy. This issue point out the necessary to identify other suitable cell type to target FVIII expression in order to induce tolerance to the transgene. At this regard, encouraging results in mice were obtained by restrict FVIII expression in platelets with the use of megakaryocytic specific promoters. Doing so, FVIII was released after platelets degranulation only in the site of injury when occur, otherwise no circulating proteins are present to trigger immune response (177). Indeed, *ex vivo* BM transplantation transduced with a LV carrying FVIII under the control of the platelet αIIb gene promoters correct the bleeding phenotype of hemophilic mice by several coagulation assay without neutralizing antibodies detection (177, 178). More important, this strategy has proven effective also in presence of pre-existing anti FVIII antibodies (179, 180). Platelets targeting provide good results also in dog and in immunodeficient mice transplanted with genetically modified human HSC (181, 182). Another approach to overcome antibodies development is to target FVIII expression specifically in organs or cell types with immuno-tolerant properties. LSEC represent an optimal candidate at this regard. In fact it has been demonstrated their attitude to induce tolerance against antigen presented by themselves (183). Moreover, because the interaction between FVIII and vWF is crucial for the stability and activity of FVIII, endothelial cells and megakaryocytes, that are the main vWF producers, represent an interesting cell target for cell and gene therapy (50). Other than promoting FVIII production in specific cell type, immune response can be reduced by abrogates transgene expression in antigen presenting cells. This was obtained by using the miRT142-3p that provided a sustained FVIII expression in mice without antibodies formation (173). A further target examined for FVIII expression in hemophilia A gene therapy application was BM-derived stem cells, in particular HSC. At this point both LV and Retroviral vectors were employed in *ex vivo* gene therapy. Interestingly, this approach induced FVIII expression and seemed to be functional to induce tolerance to FVIII by reducing inhibitors even in presence of pre-existing antibodies or after rFVIII challenging (184-187). However, the best results in these experiments were achieved using a porcine FVIII that is expressed 10-14 fold better than the human (185). FVIII expression was also drive specifically to B cells using an immunoglobulin heavy chain promoters obtaining sustained long term levels of FVIII with bleeding phenotype correction (188).

FVIII codon optimization

Beyond immunological issue, HA gene therapy is hampered by the inefficient expression of FVIII. To overcome the problem many modifications were done on FVIII coding sequence in

order to improve the expression, the intracellular maturation and the secretion pathway of FVIII. For instance, deletion of the hFVIII B-domain, which is not required for co-factor activity, resulted in a 17-fold increase in mRNA levels over full-length wild-type FVIII (30). Nevertheless secretion of BDD-FVIII is impaired in comparison of full size due to the absence of crucial asparagin-link oligosaccharides on B domain, in fact at protein level the absence of B domain provide only 30% more secretion than the wild type. This obstacle was overcome by Pipe and colleagues maintaining the proximal 226 amino-acid portion of the B-domain (FVIIIN6) that is rich in asparagine-linked oligosaccharides (189). This retention led to a 5-10 fold FVIII secretion by promoting a better interaction of FVIII with the LMAN1/MCFD2 complex that is involved in the FVIII ER-Golgi transition. Additionally, it has been described that a 110 aa region within the FVIII A1 domain contains sequences able to bind the immunoglobulin-binding protein (BiP) causing reduction in secretion by entrapping FVIII in the ER (190). At this regard it was reported that the single amino acid F309S mutation increase FVIII secretion up to 3-fold by reducing the binding of BiP with subsequent lower FVIII retention in the ER (191). Recently, other single amino acid changes were identified to improve FVIII secretion. The combined 1899G/C1903G mutations increase 2,2 fold FVIII secretion by eliminating a dispensable disulfide group (192) and more recently, Siner et al. showed that a minimal modification in the retained B domain sequence, the mutation R1645H, is sufficient to increase FVIII secretion by 2,4 fold (193). The latest mutation was setup by comparing the amino acid sequence of hFVIII with cFVIII that is more efficiently expressed and is more stable in circulation. This is due in part by the fact that cFVIII is mainly secreted as single chain whereas the human as heterodimeric form. This difference it could be explained through the different intracellular cleavage of FVIII by the protease-paired basic amino acid cleaving enzyme (PACE)/furin. cFVIII escapes from furin cleavage because in comparison with other mammalian FVIII has a unique furin consensus sequence (HXXR instead of RXXR) that confers resistance to the protease activity (193). Finally, another two fold improvement in potency was achieved by replacing the 226 amino-acid N6 spacer with a novel Asparagine reach peptide of 17 aminoacids (V3) (165).

Materials and Methods

Bladder epithelial cells isolation and culture

To analyze the presence of FVIII from healthy and tumoral bladder, specimens were isolated from several cystectomies in collaboration with Prof. Valente and Dr. Volpe from which informal consent was obtained by the patients. Bladder fragments were snap frozen in liquid nitrogen, disaggregated and lysed for mRNA extraction. Epithelial cells were isolated through mechanical removal of the bladder epithelium from stroma. Cells were maintained in culture in serum free EpiLife Medium (Life Technologies) containing Human Keratinocyte Growth Supplement (HKGS) (Life Technologies) and presence of FVIII mRNA and protein expression were assessed by RT-PCR and immunofluorescent stainings at different cell passages.

Human and mouse cells isolation, culture and differentiation

Human. Human liver samples were perfused as previously described (82). After liver dissociation with a cell scraper, cells were passed through Dacron fabric with 80-μm pores and centrifuged twice under 50 g for 5 minutes to separate hepatocytes from non-parenchymal cells (NPCs). NPCs were washed and pelleted under 350 g for 10 minutes. Human LSECs were selected from NPC fraction by immunomagnetic sorting incubating NPCs with anti-human CD31 biotin-conjugated (eBioscience) for 20 min at 4°C followed by an incubation with Streptavidin MicroBeads (MiltenyiBiotec) for 15 min at 4°C and finally isolated by MS Separation Columns (Miltenyi Biotec), according to the manufacturer's protocol. Isolated LSECs were plated at a density of 1.5-2x10^5 cells/cm^2 and cultured on collagen-coated tissue culture dishes in EGM-2 medium (Lonza). Human Kupffer cells (KCs) were isolated by plastic adhesion from NPCs after LSEC isolation. Briefly, CD31-negative fraction of NPCs was resuspended in serum-free RPMI and plated on plastic tissue culture dishes. After 45 minutes of incubation at 37°C supernatant was removed, plates were washed twice in PBS and finally fresh RPMI containing 10% fetal bovine serum (FBS) and 10ng/ml rhM-CSF was added. KCs were cultured for up to 3 weeks under these conditions. To isolate human peripheral blood mononuclear cells (PBMCs), blood was layered on Ficoll-Paque TM (PREMIUM, GE Healthcare). PBMCs were seeded in serum free RPMI medium to promote monocytes adhesion. After 30 minutes the medium was changed with RPMI containing 5% FBS and cells cultured for 12h. To obtain macrophages, monocytes were cultured in DMEM with 10% FBS, 1mM sodium pyruvate, 1mM non-essential amino acids, 0,25mM HEPES (Lonza), 10ng/ml M-CSF.

Human HSC were isolated from cord blood obtained according to an approved protocol by the Ethical Committees of UPO, Novara, Italy. First, cord blood mononuclear cells (CBMCs) were

obtained by density gradient stratification of blood on Ficoll. Than CD34+ cells were isolated from CBMCs by immunomagnetic positive selection using CD34 MicroBead Kit (MiltenyiBiotec). To obtain macrophage differentiation from HSC 10^5 CD34+ cells/ml were plated in STEM-SPAM medium (STEMCELL Technologies Inc.) containing 20% FBS, 2mM glutamine, 50 U/ml penicillin,50 µg/ml streptomycin, 30 ng/ml interleukin 3, 30 ng/ml M-CSF, 30 ng/ml Flt-3 ligand, 25 ng/ml SCF, as previously described (194). Medium was changed every 2 days. After 14 days cells acquired macrophage morphology.

Megakaryocytes were differentiated by culturing $1.5x10^6$ CD34+ cells/ml in STEM-SPAM medium containing 2mM glutamine, 50 U/ml penicillin,50 µg/ml streptomycin, 10 ng/ml interleukin 6, 10 ng/ml interleukin 11 and 20 ng/ml thrombopoietin (TPO). The medium was changed at day 3, 7 and 10 (195). After 13 days, megakaryocytes were harvested and used for experiments.

Mouse. Murine monocytes were obtained from total peripheral blood. After removal of red blood cells using red blood lysis buffer (RBLB) (150mM NH_4Cl,10mM $NaHCO_3$, 1 mM disodium EDTA) total white cells were plated at the density of $1.5\text{-}2x10^6$/ml in DMEM containing 10% FBS. After 24 hours, monocytes were attached to the plate.

BM cells were flushed from tibias and femurs of 8-9 weeks-old wild type (WT) or HA mice with DMEM containing 5% FBS. After RBC lysis $0.8\text{-}1x10^6$ of total BM cells/cm^2-plastic were differentiated in macrophages (BMDMs, bone marrow-derived macrophages) by culturing cells in IMDM containing 10% FBS and 5 ng/ml recombinant mM-CSF in vitro. For studies, BMDMs were released 5-7 days later by Versene (Gibco).

For mouse MK differentiation, c-Kit+ cells were isolated using the mouse CD117 MicroBeads kit (Miltenyi Biotech) from total BM cells obtained as described above. Isolated cells were cultured for 2 days in Stem Span containing 20 ng/ml SCF. Then 10^6 cells/ml were cultured for 3-4 days in Stem Span containing 100 ng/ml TPO, 10 ng/ml IL-6 and 10 ng/ml IL-11. All cytokines were purchased from Immunotools.

pF8 cloning in LV transfer construct

SpF8 and LpF8 were amplified by PCR from human genomic DNA by inserting at 3' and 5' ends the restriction sites for the enzymes XhoI and AgeI. These sites were used to inserted F8 promoter in place of ubiquitous PGK promoter in LV.PGK.GFP in order to obtain the LV.SpF8.GFP and LV.LpF8.GFP. To generate LV.pF8.FVIII we inserted the BDD-FVIII in place of GFP in the LV.pF8.GFP. For cloning SalI and AgeI endonucleases were used to excide

both GFP and FVIII from LV.SpF8.GFP and LV.PGK.FVII, respectively. Ligase product identity was assessed by restriction analysis and sequencing. Primers used for cloning and sequencing are reported in Table 1.

Lentiviral Vectors production

Third-generation LV were product using published protocoll (196). 293T cells were cotransfected with four plasmids by calcium phosphate precipitation; these vectors were the pMDLg/RRE packaging plasmid (12,5 ug); the pMD2.VSV-G envelope-coding plasmid (9 ug); pRSV-Rev (6,25 ug) and transfer vector plasmid LV.PGK.GFP, LV.SpF8.GFP and LV.LpF8.GFP (28 ug). All four plasmids were added to cells in a 15-cm dish and thirty hours following transfection the culture supernatant, containing the packaged viral particles, was collected and concentrated by ultracentrifugation. Collected viral particles were titrated on 293T using limiting dilution analysis. Briefly, one hundred thousand 293T were cultured in DMEM with progressively lower dilutions of each lentivirus (1:10, 1:100, 1:1000, and 1:10,000). For the LV expressing GFP, each dilution was quantified by FACS as percentage of GFP+ cells. Calculation from the titration analysis indicated about 1 -2 X 10^9 transducing viral particles per milliliter. Instead, for LV not expressing GFP, genomic DNA was isolated from 293T and titer was calculated by qPCR.

Genomic DNA isolation and LV titration by qPCR

Genomic DNA was isolated from transduced 293T cells with ReliaPrepgDNA Tissue MiniPrep System (Promega). The quantitative real time PCR was carried out in a 20-ul total volume containing 1X SYBR green PCR master mix (PROMEGA), 1 uM forward and reverse primers (wpre-Δnef) and 1 µM forward and reverse primers (h GAPDH), 50 ng of genomic DNA. Quantitative PCR were performed by incubation at 95°C for 3 minutes and 40 amplification cycles of 95°C for 3 minutes and then 60°C for 30 seconds. Primers used are reported in Table 1

Animals

Animal studies were performed according to an approved protocol by the Animal Care and Use Committees of UPO, Novara, Italy. *In vivo* experiments were performed on 8-10 weeks old mice. For GFP expression studies, LVs were delivered in C57Bl/6 WT mice. C57Bl/6 Hemophilia A mice were used for *in vivo* and *ex vivo* gene therapy studies using LV.SpF8.FVIII. Immunocompromised NOD/SCID-γNull HA mice (NSG-HA) were generated in our laboratory by crossing NOD/SCID HA mice with NOD.Cg-*PrkdcscidIl2rgtm1Wjl*/SzJ (γNull) purchased by

Jackson lab (197). For HSC transplantation studies busulfan myeloablation was performed on recipient mice. The busulfan solution for injection was prepared as follow: 25 mg of drug were solved in 1 ml of acetone and than resuspended in 9 ml of peanut oil. Immunocompetent HA mice were lethally conditioned by intraperitonal injection of 25mg/kg of busulfan from days -4 to -1 before transplantation while NSG-HA mice received a sublethal conditioning by only one injection of 50mg/kg of busulfan the day before transplantation. NSG-HA mice were kept in autoclaved microisolator cages and fed with sterile food and water at the animal facilities of the Università del Piemonte Orientale. Moreover, all animals procedures made on NSG-HA mice were performed under sterile hood.

Mouse and human Hematopoietic Stem Cells isolation and transplantation.

To isolate murine HSC (lineage negative cells, Lin-) BM was flushing from femurs, tibiae and humerus of 6-8 weeks old donor mice. After red blood lysis, Lin- cells were obtained by immunomagnetic negative selection from total BM cells using Lineage Cell Depletion Kit (MiltenyiBiotec). After isolation cells were transduced with LVs at MOI 100 and cultured at density of $1x10^6$/ml in serum free STEM-SPAM medium without cytokines. Human HSC were isolated from cord blood obtained as decribed above and cultured at density of $1x10^6$/ml in serum free STEM-SPAM medium (Lonza) added with 50 ng/ml hTPO, 50 ng/ml hSCF, 50 ng/ml hIL-3 and 50ng/ml hFlt3-L. On the basis of experiment CD34+ cells were transduced with LV at MOI 30. For transplantation, 24h after isolation a total of 3 $x10^5$ or $6x10^5$ CD34+ or 10^6 lin- cells were resuspended in serum free STEM-SPAM without cytokines and tail vein injected in 400µl of volume in busulfan-conditioned mice.

Analysis of blood and organs of treated mice.

The engraftment of transplanted mice was evaluated at several time point in the peripheral blood as percentage of GFP+ or human CD45+ cells. For each time point blood was collected by retro-orbital puncture using a glass capillar. Eritrocytes were eliminated by incubating RBLB for 10 min at 4°C. Total white cells were directly analyzed by cytofluorimetry for GFP or incubated with anti-human CD45 PE conjugated antibody to assess the engraftment after xenotransplant. Total spleen cells were obtained by spleen digestion for 30' at 37°C in HBSS (Sigma Aldrich) containing 10% FBS and 0.2 mg/ml collagenase IV and then filtered through a 70-µm cell strainer (Falcon). BM cells were obtained by flushing tibiae and femurs. For both spleen and BM red blood cells were lysed for 8' with RBLB. Thymus were mechanically disrupted through a 70-

μm cell strainer. For the liver, hepatocytes and liver non parenchymal cells (NPC) were separated after liver perfusion as previously described (82). Briefly, liver was perfused via portal vein with buffer at 37°C containing 1.9 mg/ml EGTA, for 2' with buffer lacking EGTA, and for 7–9' with buffer containing 0.03% (w/v) collagenase and 5 mM CaCl2.2H2O. The liver was dissociated in perfusion buffer, and cells were passed through 80-μm filter pores and centrifuged twice at 50 g for 5' to isolate hepatocytes. NPCs in the supernatant were washed and pelleted under 350 g for 10'.

Flow cytometric analysis

For cell staining cells were resuspended in staining buffer (PBS, FBS 0,5% and NaN_3 0,1%) and incubate for 10' with anti-mouse CD16/CD32 as blocking and finally incubated for 30' on ice. The antibodies used were listed in Table 2. For the acquisition a FACS Calibur has been used (Becton Dickinson Immunocytometry System) and data obtained were analyzed by Flowing 2.5 software (Cell Imaging Core, Turku Centre for Biotechnology, Finland).

RT-PCR

RNA was isolated by Isol-RNA Lysis Reagent (5PRIME). 1 μg of total RNA was reverse-transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and PCRs were performed on cDNA. All the PCRs were performed with GoTaq® Flexi DNA Polymerase (Promega). PCR protocol were as follow: initial denaturation at 95°C for 5' followed by 30 cycles (25 cycles for β-actin) of denaturation at 94°C for 30", annealing at 54-62°C for 30-45", extension at 72°C for 60", and final extension at 72°C for 7'. Primers, annealing temperatures and product sizes are listed in the Table 1. PCR products were resolved in 2% agarose gels

Immunofluorescence

For cell stainings both human and mouse cells were plated on 12 mm Ø dish glass at concentration of $2x10^4$, the same number of megakaryocytes were cyto-spinned at 1000 rpm for 5'. Cells were fixed in PAF 4% for 5', permeabilized in 0,5% PBS-TritonX100 for 7' and then incubated with blocking buffer (5% goat serum, 1% BSA, 0.1% Triton X-100 in PBS) for 1h at room temperature (RT). For mouse organs stainings, liver and spleen of injected mice were fixed for 2h in paraformaldeidehyde (PAF) 4%, equilibrate in sucrose 30% for 48h and finally embedded in optimal cutting temperature medium (OCT). 5-6 μm thick cryostat sections were post-fixed in PAF 4% before blocking. Human paraffin-embedded bladder specimens were

obtained from pathology division. Before staining procedure, 5- to 6-μm thick paraffin-embedded bladder sections were treated in boiling 50mM EDTA pH 8 for antigen retrieval using a microwave oven. Primary antibodies were solved in PBS containing 2% goat serum, 1% BSA, 0.1% Triton X-100, and incubated for 1h at RT. After washing, sections were incubated with the secondary antibodies (in PBS containing 1% BSA, 0.1% Triton X-100) for 45' at RT and finally samples were mounted with Moviol (Sigma Aldrich). For nuclei detection DAPI or TOPRO were added to the secondary antibodies solution. Primary and secondary antibodies used and dilution are reported in Table 3. For FVIII staining a rabbit polyclonal antibody previously generated in our lab were used. The high sensitivity and specificity of the antibody was confirmed by ELISA using FVIII immunogen. The antibody recognized endogenous hFVIII using liver as a positive control. As negative control in IF cells or tissues were incubated with the preimmune serum of rabbit in which the antibody was developed (NRS, normal rabbit serum)

FVIII activity assays

FVIII activity was measured on treated mice plasma by activated partial thromboplastin time (aPTT) assay, chromogenic assay using Coatest® SP4 FVIII kit (CHROMOGENIX) and tail clip assay at different time point. To obtain the plasma from mice, peripheral blood was collected in 3.2% citrate and centrifuged at 3000 rpm for 10'. Standard curves were generated by serial dilution of a commercial hFVIII (KOGENATE®, Bayer) for chromogenic assay and by serial diluition of pooled hemophilic mouse plasma in human plasma for aPTT assay. Results were expressed in IU and percentage of correction for chromogenic and aPTT, respectively. Tail clip assay was performed on anesthetized mice by cutting the distal portion of the tail at a diameter of 3-4 mm; the tails were then placed in a conical tube containing 14 ml of saline at 37 °C and blood was collected for 2'. Tubes were centrifuged to collect erythrocytes, resuspended in red blood lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA), and the absorbance of the sample was measured at wavelength 575 nm. Result was analyzed by comparing the amount of blood loss obtained from treated HA mice with WT and untreated HA mice serving as controls.

Anti-FVIII antibodies detection

Direct ELISA was performed on plasma of treated mice to evaluated the presence of anti FVIII antibodies. 96 wells plates were coated over night at 4°C with 5µg/ml of commercial recombinant BDD-FVIII (Refacto, Pfizer), after that wells were washed and saturated with BSA (TBS-BSA 0,2%) for 3 hours at 37°C. Plasma of injected mice was serially diluted from 1/200 to 1/2000 and incubated as primary antibody for 2 hours at 37°C. After washing, the secondary antibody (sheep anti-mouse horseradish peroxidase conjugated antibody) was incubated for 1 hour at 37°C. Reaction was developed with TMB (tetramethilbenzidine) prepared according to the manifacturer's protocol and stoped with H_2SO_4 0,5 N. Absorbance were finally analyzed by spectophotometer at 450nm wavelength. Results were normalized using a pooled plasma of non injected HA mice and were expressed as Arbitrary Unit (A.U.)

Statistical analysis

All data were expressed as mean ± SD. P-value were calculated using Student's T test analysis. $P< 0,05$ was considered statistically significant (*), $P<0,01$ very significant (**).

Table 1: primers used in PCR, RT-PCR, sequencing and qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	T annealing	Product size (bp)
hβ-actin	gagaaaatctggcaccacacc	cgacgtagcacagcttctc	56°C	412
mβ-actin	gtggggcgccccaggcacca	cttccttattgtcacgcacgatttc	56°C	540
hFVIII	ggagagtaaagcaatatcagatgc	ggtgaattcgaaggtagcgac	56°C	389
mFVIII	ggtatcaaagtgacaatgtacc	ccaattaatcccgagtgcatatc	54°C	394
mGPαIIβ	cagggccaagtgctgatatt	ttgaagcagctgactggtgt	54°C	302
mvWF	acagacgccatctccagattca	tgttcatcaaatggtgggcagc	62°C	272
SpF8 amplification	gaatctcgagcagcagttcccaca aacgttaccc	cgcaccggtgacttattgctacaa atgttcaac	60°C	1175
LpF8 amplification	gaatctcgagcagcagttcccaca aacgttaccc	cgcaccggtgacttattgctacaa atgttcaac	60°C	2350
pF8 sequencing 1	ctgtcattcccaggggttgg		60°C	
pF8 sequencing 2		cccacctgcatcactgatatc	60°C	
pF8 sequencing 3		cgtcgccgtccagctcgaccag	60°C	
LV integration qPCR (Wpre forward, dNEF reverse)	tggattctgcgcgggacgtc	ggctaagatctacagctgccttg	60°C	216
hGAPDH qPCR	agtgggtgtcgctgttgaagt	aacgtgtcagtggtggacctg	60°C	180

Table 2: Antibodies used for FACS

Antibody	Reactivity	Manufacturer	Format	Incubation condition
CD16/32	mouse	BD Pharmingen	Purified	10 min on ice
CD45	human	Miltenyi Biotec	PE	30 min on ice
CD19	human	Miltenyi Biotec	PE	30 min on ice
CD14	human	Immunotools	FITC	30 min on ice
CD3	human	Immunotools	FITC	30 min on ice
CD33	human	Immunotools	FITC	30 min on ice
CD11b	human/mouse	Miltenyi Biotec	PE	30 min on ice
Tie-2	human	Miltenyi Biotec	PE	30 min on ice
CD31	human	Immunotools	APC	30 min on ice
CD14	mouse	Biolegend	APC	30 min on ice
ScaI	mouse	e-Bioscience	PE	30 min on ice
Gr-1	mouse	Immunotools	APC	30 min on ice
B220	mouse	eBioscience	PE	30 min on ice
CD11c	mouse	eBioscience	PE	30 min on ice
CD4	mouse	Immunotools	PE	30 min on ice
CD8a	mouse	Immunotools	APC	30 min on ice
CD19	mouse	Miltenyi Biotec	PE	30 min on ice
CD3	mouse	Miltenyi Biotec	PE	30 min on ice
F4/80	mouse	Invitrogen	PE	30 min on ice
Tie-2	mouse	eBioscience	PE	30 min on ice
CD31	mouse	eBioscience	APC	30 min on ice
CD146	mouse	Miltenyi Biotec	PE	30 min on ice

Table 3: Antibodies used for immunofluorescence

Primary antibodies	Host	Reactivity	Manufacturer	Dilution	Incubation condition
CD31	mouse	human	BD pharmingen	1:200	1h at RT
vWF	rabbit	human/mouse	Sigma-Aldrich	1:300	1h at RT
vWF	mouse	human	Santa Cruz Biotechnlogy, inc.	1:100	1h at RT
CD146	mouse	human	Miltenyi Biotec	1:300	1h at RT
CD11b	mouse	human	Immunotools	1:100	1h at RT
CD14	mouse	human	Immunotools	1:100	1h at RT
CD61	rabbit	human	Santa Cruz Biotechnlogy, inc.	1:100	1h at RT
CK7	mouse	human	Dako	1:500	1h at RT
FVIII	rabbit	human/mouse		1:300	1h at RT
GFP	rabbit		Molecular Probes	1:1000	1h at RT
F4/80	rat	mouse	AbD serotec	1:500	1h at RT
CD31	rat	mouse	BD pharmingen	1:75	1h at RT
Secondary antibodies	**Fluorophores**				
Goat anti-rat IgG	Alexa Fluor 546		Molecular Probes	1:500	45-60 min at RT
Goat anti-rabbit IgG	Alexa Fluor 546 or 488		Molecular Probes	1:500	45-60 min at RT
Goat anti-mouse IgG	Alexa Fluor 546 or 488		Molecular Probes	1:500	45-60 min at RT

Results

PART I

Extrahepatic sources of FVIII

FVIII expression in human liver cells

Despite the discovery of extrahepatic sources of FVIII, liver is the main organ producing FVIII, however the identity of liver cell type expressing FVIII is controversial. Recently, LSEC and not hepatocytes were shown to be the main source of FVIII in liver (77-79). To confirm these data in human cells, we isolated LSEC, hepatocytes and Kupffer cells (KC) from liver biopsies. FVIII expression was found by IF in cultured LSEC with co-staining for LSEC markers such as CD31, CD146 and vWF (Fig. 1A). Among non-parenchymal cells, FVIII was also detected in CD11b+ KC (Fig.1 B-D). Interestingly, human hepatocytes were positive for FVIII staining, but at a lower level and not in all cells compared to LSEC (Fig. 1E). As FVIII negative control for each staining cells were incubated with the normal rabbit serum (NRS) (Fig. 1A,D,E) The identity of isolated and cultured human LSEC and KC was verified by FACS using Tie-2 and CD31 markers for LSEC and CD11b and CD14 markers for KC (Fig. 1F). By qPCR we confirmed IF results that human LSEC have at least 5 times more FVIII mRNA than hepatocytes and KC ($p<0,05$) (Fig. 1G). These experiments demonstrate the significant role of LSEC in FVIII production without excluding the contribution of other liver cells for FVIII synthesis.

FVIII expression in cancer cell lines

To extend our knowledge on cells able to express FVIII we analyzed data regarding FVIII expression coming from a transcriptome analysis of 909 cancer cell lines. First, all cell lines were clustered according to the origin and cancer type and then each cluster was stratified on the basis of the number of cell lines that showed high and low level of FVIII and for the average of expression (mean of expression; MOE). We select 7.1 as the mean average of expression to consider high and low FVIII expressing cell lines. Among the clusters that exhibited the majority of cell lines with an average higher than the threshold there are Hodgking disease (87,5%), chondrosarcoma (83,3%) and myeloid (65,4%) (Fig. 2A), however data regarding Hodgking disease and chondrosarcoma cluster could be biased by the sample numerosity (8 and 6 cell lines per cluster respectively) affecting the statistical significance of the results. Furthermore, myeloid

cells is the second best cluster by analyzing the MOE with a score of 7.34 (Fig. 2B) and showed the highest average if we consider only the high FVIII expressing cell lines (8,44) (data not shown). This data is in line with what we have previously reported showing the ability of hematopoietic cells to express FVIII and to correct bleeding phenotypes in HA mice after total BM transplantation in mice (93). Regarding other hematopoietic cancer cell lines, compared to myeloid, lymphoid cluster displayed lower FVIII expression score in term of both number of high FVIII expressing cell lines (33,92%) and MOE (7,07) (Fig. 2A,B). However, by a further stratification of lymphoid cluster, T cell lines showed very high score of FVIII expression in comparison with B cells (Fig. 2C,D) and this explain the fact that FVIII cDNA was firstly cloned from a T cell line (97). Conversely, among the clusters showing low FVIII expression there are endometrium, which displayed only 12,5% of cell lines with a score >7.1, gastric (17,65%), colon (21,31%) and interestingly liver/hepatocytes (25,93%) (Fig. 2A) showing the lowest MOE score (6,39) (Fig. 2B). The latter data support previous observation regarding FVIII expression in liver cells by qPCR (Fig,1G) which proved low FVIII mRNA amount in hepatocytes.

FVIII expression in bladder

During a survey on FVIII expression in several organs and tumours in collaboration with professor Valente (Anatomy-Pathology in the dep. of Translational Medicine), we detected by immunohistochemistry a remarkable amount of protein in both normal and cancer bladder specimens. Moreover, RT-PCR analysis performed previously in the lab showed the presence of FVIII mRNA in ECV, a bladder epithelium cell line. To elucidate these preliminary data, we analyzed the presence of FVIII antigen in different histological bladder samples. By IF the presence of FVIII was detected in a reproducible manner in all analyzed samples with an apparent increase in cancer tissue (Fig. 3B) compared with normal (Fig. 3A); further, epithelial cells represent the main cells producing FVIII as showed by the co-staining between FVIII and cytokeratin 7 (CK7), a typical bladder epithelial marker (Fig. 3A,B). To explore this aspect, we collected from the urology surgeons several fragments of normal and tumor tissues from cystectomies. RT-PCR experiments on mechanically fragmented bladder fragments showed the presence of FVIII mRNA. Particularly, in three samples we observed a band of greater intensity in the tumor tissues compared to apparently healthy tissue from the same cystectomy (Fig. 3C compare lanes 3-4, 7-8, 9-10). Interestingly, no FVIII mRNA at all was detected in a sample that when analyzed by the pathologist showed absence of epithelium (Fig. 3B lane 2). To better investigate FVIII expression in CK7+ cells a piece of each bladder was collected to isolate and

culture epithelial cells (BEC). From 4 bladders we successfully isolated polygonal-shaped cells only from healthy tissue showing ephitelial cells morphology (Fig. 3D). In the best case, cells were maintained in culture for more than a month and up to passage 8 before they became senescent. The mRNA was isolated from cells at different passage and it was always possible to detect FVIII mRNA by RT-PCR (Fig. 3E). We also analyzed the presence of FVIII protein in bladder epithelial cells by IF. Co-staining of FVIII and CK7 was present in these cells and the localization of FVIII in positive cells was perinuclear and with a dotted appearance (Fig. 3F).

FVIII expression in hematopoietic cells

The discovery that BM transplantation from healthy mice to hemophilic recipients is able to correct the bleeding phenotype in HA focused our interest on hematopoietic cells as an extrahepatic source of FVIII. Thus, we explored, both in human and mouse, which hematopoietic cell population mainly expressed FVIII. Peripheral blood monocytes and monocyte derived-macrophages (MDM) expressed FVIII at mRNA and protein levels (Fig. 4). However, human MDM showed greater FVIII expression by RT-PCR compared with freshly isolated monocytes (Fig. 4A compare lanes 2-3, 4-5). Otherwise, the FVIII mRNA level in lymphocytes or neutrophils was very low compared to monocytes and macrophages (Fig. 4A). In cells from HA mice FVIII was absent (Fig. 4B lanes 2-4) and was found only in monocytes of WT mice (Fig. 4A lane 5-7). IF demonstrated FVIII in monocytes as well as in MDM and dendritic cells (Fig. 4C) with co-staining of FVIII and CD14 monocyte marker (in human cells) (Fig. 4C) or FVIII and F4/80 monocyte/macrophage marker (in mouse cells) (Fig. 4D). Similar results were obtained in differentiated macrophages from mouse BM or CD34+ hCB cells. After adhesion to plastic, these differentiated macrophages displayed a typical ”fried egg” morphology, abundant cytoplasm CD11b and CD14 markers (Fig. 5A-C). RT-PCR confirmed that human hematopoietic stem cells and CD34+ derived-macrophages (DM) expressed FVIII mRNA (Fig. 5D) and similarly in mouse, FVIII mRNA was present in total BM, mainly in c-Kit+ cells as well as in BM derived-macrophages (BM-DM) (Fig. 5E). These results were confirmed by co-staining of FVIII with CD14 in human cells (Fig. 5F) and of FVIII with F4/80 in mouse cells (Fig. 5G).

In view of the physiological role of vWF in stabilizing plasma FVIII, we investigated whether MK could possibly express FVIII. Human CD34+ or mouse c-Kit+ cells were differentiated into MK, becoming larger with rounded morphology (Fig. 6A,B). Further, in mouse MK differentiated from c-Kit+ cells vWF and GpαIIβ mRNAs were up-regulated after cytokine stimulation (Fig. 6C). Both human and mouse MK expressed FVIII at mRNA (Fig. 6D, E) and

protein levels, as shown by IF stainings by co-expression of FVIII and the specific megakaryocyte markers such as human CD61 and vWF (Fig. 6F, G).

In vivo studies in hemophilia A mice

Human CD11b+ cells secrete functional FVIII and correct hemophilia A in NOD-SCID HA mice.

Because of the complexity of post-translational modification and trafficking of FVIII in cells, it is crucial to determine whether FVIII-expressing cells may actually secrete the functional protein. To evaluate this property in CD11b+ human monocytes, we transplanted by tail vein injection 11 NOD-SCID HA mice with 15 $x10^6$ CD11b+ CB-derived cells. The presence and biodistribution of transplanted human CD14+ cells was assessed in recipient mice blood, spleen and liver 1 week after transplantation. In peripheral blood and spleen we found up to 3% of human cells as detected by FACS analysis (Fig.7A, B). Cells engrafted in the liver, as shown in IF staining (Fig. 7C). The FVIII activity was measured in the plasma of treated mice 3 and 7 days after cell infusion. Four of the transplanted mice showed detectable FVIII activity by Coatest assay (Fig. 7D). After one week, 9 out of the 11 treated mice survived after tail clip assay, while all control mice died (Fig. 7F). These results were confirmed in NOD/SCID γ-null HA (NSGHA) mice injected with the same number of CD11b+ cells and killed one week later. FACS analysis on NSGHA spleen and blood confirmed the presence of human cells (not shown). FVIII activity measured by aPTT assay confirmed the results obtained in NOD/SCID HA mice (Fig. 7E). In conclusion, injected human monocytes secreted FVIII and rescued the bleeding phenotype in HA mice.

Human CD34+ cell transplantation correct hemophilia A in NSG-HA mice.

Given the results of FVIII expression in hematopoietic cells and particularly in myeloid cells, we transplanted human CD34+cells in recipient immunodeficient NSG-HA mice to verify the extent of phenotypic correction following BM replacement with CB derived cells. The experimental design included one group of mice receiving $3x10^5$ CD34+ cells (low dose group, LD) (n=6) and a group of mice that was injected with $6x10^5$ CD34+ cells (high dose group, HD) (n=6). Cell engraftment of transplanted mice was analyzed by FACS analysis 8 and 12 weeks after CD34+ injection as percentage of human CD45+ hematopoietic cells in blood samples. After 8 weeks the

mean percentage of human circulating cells was 39,3±22,7% in LD and 25,3±11,4% in HD (Fig. 8A). Interestingly, the higher cell engraftment was observed in the low dose group of transplanted mice. Percentage of human CD45+ cells was maintained until 12 weeks (36,8±16,8 and 25,4±9,1) (Fig. 8B) when mice were challenged by tail clip survival assay to assessed the phenotypic correction. FVIII activity was analyzed 12 weeks after transplantation by aPTT, showing that FVIII activity was superior than 2% in most of transplanted mice, sufficient to ameliorate bleeding phenotype (Fig. 8C). In particular we detected an higher FVIII activity in the high dose group of transplanted mice than in the low dose group, 3.7±1.3 and 2.4±1.1 ($p<0,05$) respectively (Fig. 8D). To assess the correction of the bleeding phenotype transplanted mice were challenged by tail clip survival assay. 9 Out of 12 challenged mice (75%) survived after the tail clip, while all control mice died (Fig. 8E). Interestingly, survived mice showed FVIII activity of 3,6±1,2 by aPTT corresponding to the higher correction obtained in vivo with a clear correlation between FVIII levels and survival (data not shown).

At the end of the tail clip assay, human cell engraftment in spleen and BM was evaluated. The percentage of human CD45+ cells was similar in both groups receiving low and high cell dose (BM: 63.6±6.5 and 54.5±8.5; spleen: 62.2±17.2 and 62.5±2.3) (Fig. 8A,B). Myeloid differentiation of transplanted cells was investigated in BM by staining with CD33 and CD14 antibodies founding more CD14+ than CD33+ cells (Fig. 8A). In the spleen of transplanted mice, human cells were predominantly B cells, 40% were CD19+, while CD3+ cells were <8% and monocytes were <20%, as shown by CD14 and CD11b staining. Engrafted human cells were more often CD11b+ than CD14+ (Fig. 9B). These data emphasized the role of BM-derived cells as extrahepatic source of FVIII and making hematopoietic cells a potential target for hemophilia A cell therapy.

PART II

FVIII promoter activity

In silico analysis of transcriptional factors recognizing FVIII promoter

Another approach to investigate FVIII cell expression and its transcriptional regulation could be represented by the study of F8 promoter. The proximal F8 promoter was previously characterized by Figueiredo at al. starting from a 1175 bp sequence initially reported in 1985. Nevertheless, since this sequence does not necessarily recapitulates the activity of the endogenous promoter we extended our study including a longer genomic sequence (2350bp) (LpF8) to compare its efficiency and cell specificity with the one described by Figueiredo (SpF8). We blasted F8 promoter sequence (both long and short) in a specific software for transcriptional factor (TF) binding site prediction (http://alggen.lsi.upc.edu/recerca/menu_recerca.htm). The dissimilarity margin between target sequences on F8 promoter and specific TF consensus sequence was taken less than 5% to provide a more precise prediction. For each TF, two parameters were considered: the number of nucleotides recognized by a specific TF and the number of consensus sequences in the F8 promoter. This analysis predicted the presence of well know hepatocytes TF such as HNF1, HNF3, C/EBPα and C/EBPβ that were also identified by Figueiredo (Fig. 10A). In addition Pea3, Ets1 and Ets2 endothelial specific TF and several myeloid-specific TF such as C/EBP, GATA1, IRF-2 and STAT1 and STAT4 were found. In particular, IRF-2 and STAT1-4 are important regulators of myeloid differentiation and activation (Fig. 10A). A part from LEF-1, in comparison with the short sequence, the long sequence did not show the presence of new TF consensus sequences (Fig. 10A).

In vitro FVIII promoter activity

To assess FVIII promoter activity *in vitro* we inserted the human long and short promoter sequences in a lentiviral transfer construct containing GFP as gene reporter (LV.LpF8.GFP and LV.SpF8.GFP). The 2 LV and the control LV which express the GFP under the control of the ubiquitous promoter PGK (LV.PGK.GFP) were used to transduce at MOI 0.2 several human cell lines of endothelial (hECV and HUVEC), hematopoietic (DAMI megakariocytic cell line, U937 monocytic cell line, jurkat T cell line and SSK41 neoplastic B cell line) and hepatic origin (HepG2 and Huh7) according to the presence of cell specific TF identified by the *in silico*

analysis. Interestingly, despite the prediction of several hepatocytes specific TF, both F8 promoters are less active in the hepatic cell lines HepG2 and Huh7 when compared with GFP expression driven by PGK promoter used as control at the same MOI (Fig. 10B). Similarly to hepatocytes, low GFP expression was observed in primary human foreskin fibroblast (HFF) after LV.pF8.GFP transduction in comparison with PGK (Fig. 10B). Meanwhile GFP expression difference between F8 and PGK promoters decreased in endothelial (hECV and HUVEC), myeloid (DAMI and U937) and lymphoid cell lines (Jurkat and SSK41) (Fig. 10B). This mean that, in vitro, pF8 drive transgene expression specifically in endothelial and hematopoietic cell lines, while it is less active in hepatocytes and unrelated cells (HFF). However, *in vitro* experiments did not show significant differences in GFP expression in transduced cells between SpF8 and LpF8. Noteworthy, pF8 activity in tested cell lines correlates with the previous transcriptome analysis on FVIII expression described above, suggesting the reciprocal validity of the data.

***In vivo* FVIII promoter activity**

Because of the similar performances between the 2 F8 promoter sequences, we decided to carry out preliminary *in vivo* studies starting with the SpF8. We injected $5x10^8$TU of LV.SpF8.GFP in C57BL/6 mice and evaluated GFP expression by FACS and immunofluorescence analysis in several organs at different time points after injection (1, 2, 4, 8, 12 and 24 weeks). In hematopoietic organs, such as spleen and BM, GFP expression was sustained up to 1 month (10-22% in BM and 4-10% in spleen) and was predominantly restricted to monocytic cells as shown by CD11b, CD11c and F4/80 co-staining (Fig. 11A). Little co-positivity was assessed in BM with cell-specific markers for B cells with B220, and granulocytes with Gr-1. While in the spleen pF8 seems to be less active in CD4 and CD8 subpopulation, more B220 GFP positive cells were detected when compared with BM (Fig. 11A). Interestingly, after 4 weeks, GFP expression in BM decreased at level lower than 2% (data not shown). In the liver, GFP expression was restricted to hepatic non-parenchymal cells (24,8±6,2%) and hepatocytes were barely detected (<2%) (Fig. 11B).

To better identify which NPC subpopulation specifically expresses GFP under the control of F8 promoter, we performed more characterizations. In particular, we evaluated the co-expression by FACS and IF of GFP and LSEC or KC-specific markers and we observed that pF8 was predominantly active in LSEC. Indeed, by FACS analysis GFP positivity was assessed mainly in cells expressing LSEC specific markers such as CD31, CD146 and Tie-2 and less co-staining was

observed in CD11b and F4/80 positive KC (Fig. 11C). This result was confirmed by IF in which we observed a strong co-expression between GFP and CD31 but very few F4/80-GFP double positive cells were detected (Fig. 12A, B). Interestingly, by IF, GFP expression resulted virtually absent in hepatocytes (Fig. 12A, B). On the contrary, when we analyzed the spleen of LV.SpF8.GFP injected mice we mainly detected GFP expression in macrophages with few GFP positive endothelial cells (Fig. 12C, D). Thus, our *in vivo* studies demonstrated that FVIII promoter is mainly active in monocyte/macrophages and endothelial cells with a particular expression pattern based on the organ analyzed. Moreover, in liver context our data confirmed results recently published that identify LSEC and not hepatocytes as main hepatic cell type expressing FVIII (77-79).

FVIII promoter activity in hematopoietic cells

In order to restrict our studies on F8 promoter activity in hematopoietic cells, we transplanted LV.pF8.GFP and LV.PGK.GFP (control) lin- transduced cells from recipients in busulfan-conditioned C57BL/6 mice. FACS analysis on transplanted mice blood starting 4 weeks after transplantation showed >15% GFP positive cells for pF8 and >60% for PGK meaning that FVIII promoter is active in BM-derived cells (Fig. 13A). Moreover, GFP expression in blood cells was stable without significant changes up to 4 months when mice were killed and organs analyzed (Fig. 13A). GFP positivity was observed by FACS at various levels in all organs examined, suggesting that F8 promoter is active also in hematopoietic cells distributed in several tissues such as BM, spleen, thymus and liver NPC (Fig. 13B). In particular compared with other organs few GFP+ cells were reported in thymus of pF8-Lin- transplanted mice, in which the predominant cell population is represented by T lymphocytes, indicating low pF8 activity in these cells (Fig. 13B). To further clarify in which hematopoietic cell type pF8 is mainly active, GFP expression in each organ was co-analyzed with several cell specific markers by FACS and IF. In the blood, pF8 driven GFP expression mostly in myeloid cells as shown by CD11b and Gr-1 co-staining, however we detected less GFP positivity in granulocytes compared with CD11b+ monocytes (Fig. 13C). This difference between granulocytes and monocytes confirmed what we observed in RT-PCR regarding FVIII expression in these cells (Fig. 4A). On the other hand, very low co-staining was assessed between GFP and lymphocytes cell specific markers such as CD19 for B cells and CD4 and CD8 for T cells (Fig. 13C). The specific activity of pF8 was further confirmed by analyzing PGK-Lin- transplanted mice blood in which GFP expression was observed without differences both in myeloid and lymphoid cells (Fig. 13C). Similarly to blood,

in BM, GFP expression driven by pF8 was detected predominantly in CD11b+ myeloid cells rather than in B lymphocytes (Fig. 13D). Interestingly, in contrast with what we observed in direct LV injection less GFP expression was detected in ScaI+ hematopoietic precursor cells (Fig. 13D). Additionally, in the spleen GFP was expressed mostly in myeloid cells (stained with CD11b and CD11c) in comparison with lymphocytes (CD3 for T cells and CD19 for B cells) (Fig. 13E). As mentioned above, low GFP expressing cells were detected in thymus, however we reported little co-staining in this organ between GFP and CD4 or CD8 lymphocytes markers (Fig. 13F). Regarding the liver, IF staining on hepatic sections of pF8-Lin- transplanted mice showed the presence of F4/80-GFP expressing cells that are less in comparison with those transduced with the PGK promoter (Fig. 14A, B). Moreover, pF8-Lin- mice were injected with 200mg/kg of monocrotaline (MCT) to induce liver endothelial damage and study monocytes migration in the liver and macrophages differentiation 10 days later. Interestingly, we observed that MCT treatment dramatically increase the presence of F4/80+ KC expressing GFP compared to untreated mice (Fig. 14C). This suggested that after liver injury and subsequent inflammation somehow a F8 promoter activation occur in macrophages. Similarly to the liver, in the spleen of pF8-Lin- mice, GFP positivity is restricted predominantly in F4/80+ macrophages whereas PGK drives GFP expression also in the germinative center rich in B cells (Fig. 14D, E). This suggests that in the spleen, pF8 is mainly active in myeloid cells in comparison with lymphocytes. Although no significant increase of GFP+ macrophages was observed in the spleen after MCT treatment (Fig. 14F). The GFP studies (both in vivo and ex vivo), strongly indicate that pF8 is specifically active in myeloid population rather than in lymphocytes.

HA gene therapy using FVIII promoter

In vivo gene therapy

In our laboratory we observed sustained FVIII activity without inhibitors formation in HA mice injected with a LV carrying the FVIII under an endothelial (VE cadherin promoter) or a monocytic promoter (CD11b promoter). Starting from these results, we were interested to investigate whether pF8 was suitable for gene therapy application because of its ability to drive in vivo transgene expression in endothelial and myeloid cells. Thus, LV.pF8.FVIII $1x10^9$ TU were tail vein injected in six C57BL/6 HA mice. aPTT assay on plasma of treated mice showed therapeutic levels of FVIII (6-10%) up to 8 months, the longest time tested so far (Fig. 15A).

Noteworthy, in contrast with mice injected with PGK.FVIII, by ELISA we did not detect anti-FVIII antibodies in the plasma of all injected mice overtime (Fig. 15B). For instance, these results encouraged further studies on F8 promoter for gene therapy applications.

***Ex vivo* gene therapy**

Combining gene therapy with HSC transplantation provided good results for HA treatment in mice, especially because it has been demonstrated to induce immunotolerance (184-187). Further, we have demonstrated that BM derived cells express FVIII and after transplant ameliorate the bleeding phenotype. At this point we wanted to verify if *ex vivo* transplantation of both human and mouse HSC transduced with pF8.FVIII reached therapeutic levels of FVIII in busulfan-treated HA mice. Human CD34+ cells were LV-transduced with MOI 30 while Lin- isolated from C57BL/6 HA mice with MOI 100. Copy number integration analysis showed a mean of 4,4 LV genome per cell for CD34 and 3,2 for Lin-. Human HSC ($6x10^5$) were then injected in 6 NSG-HA whereas 10^6 Lin- cells were administered to 3 C57BL/6 HA mice. As control in the CD34 group 2 mice were transplanted with untransduced cells. Human cells engraftment was evaluated by FACS as percentage of hCD45+ cells in blood of transplanted mice, resulting in 29±8,6 at one month, 34,5±16 at two months and 28±17 at 3 months after HSC injection (Fig. 15C). aPTT assay performed up to 3 months on plasma of pF8-CD34 transplanted mice showed therapeutic levels of FVIII activity around 8-10% of normal meanwhile transplantation of untransduced CD34+ cells reached 2% of activity, confirming the our previous data (Fig. 15D). Similar results were assessed by transplanting LV.SpF8.FVIII-transduced murine Lin- cells. Indeed, in injected HA mice, FVIII activity was restored at 5-6% up to 3 months (Fig. 15E).

Fig.1

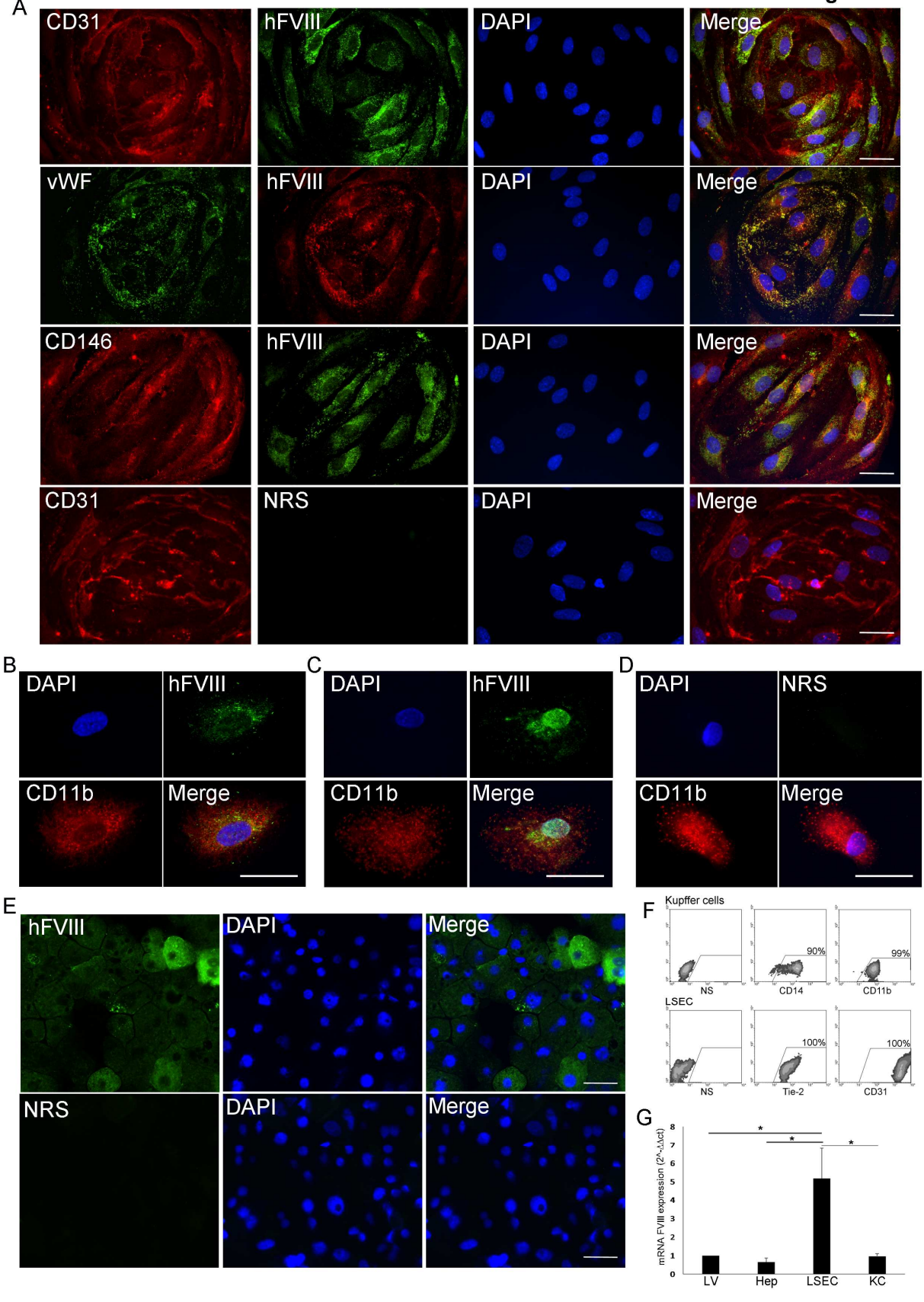

A
CD31
hFVIII
DAPI
Merge
vWF
hFVIII
DAPI
Merge
CD146
hFVIII
DAPI
Merge
CD31
NRS
DAPI
Merge
B
DAPI
hFVIII
CD11b
Merge
C
DAPI
hFVIII
CD11b
Merge
D
DAPI
NRS
CD11b
Merge
E
hFVIII
DAPI
Merge
NRS
DAPI
Merge
F
Kupffer cells
90%
99%
NS
CD14
CD11b
LSEC
100%
100%
NS
Tie-2
CD31
G
mRNA FVIII expression (2^-ΔΔct)
LV
Hep
LSEC
KC

Fig.2

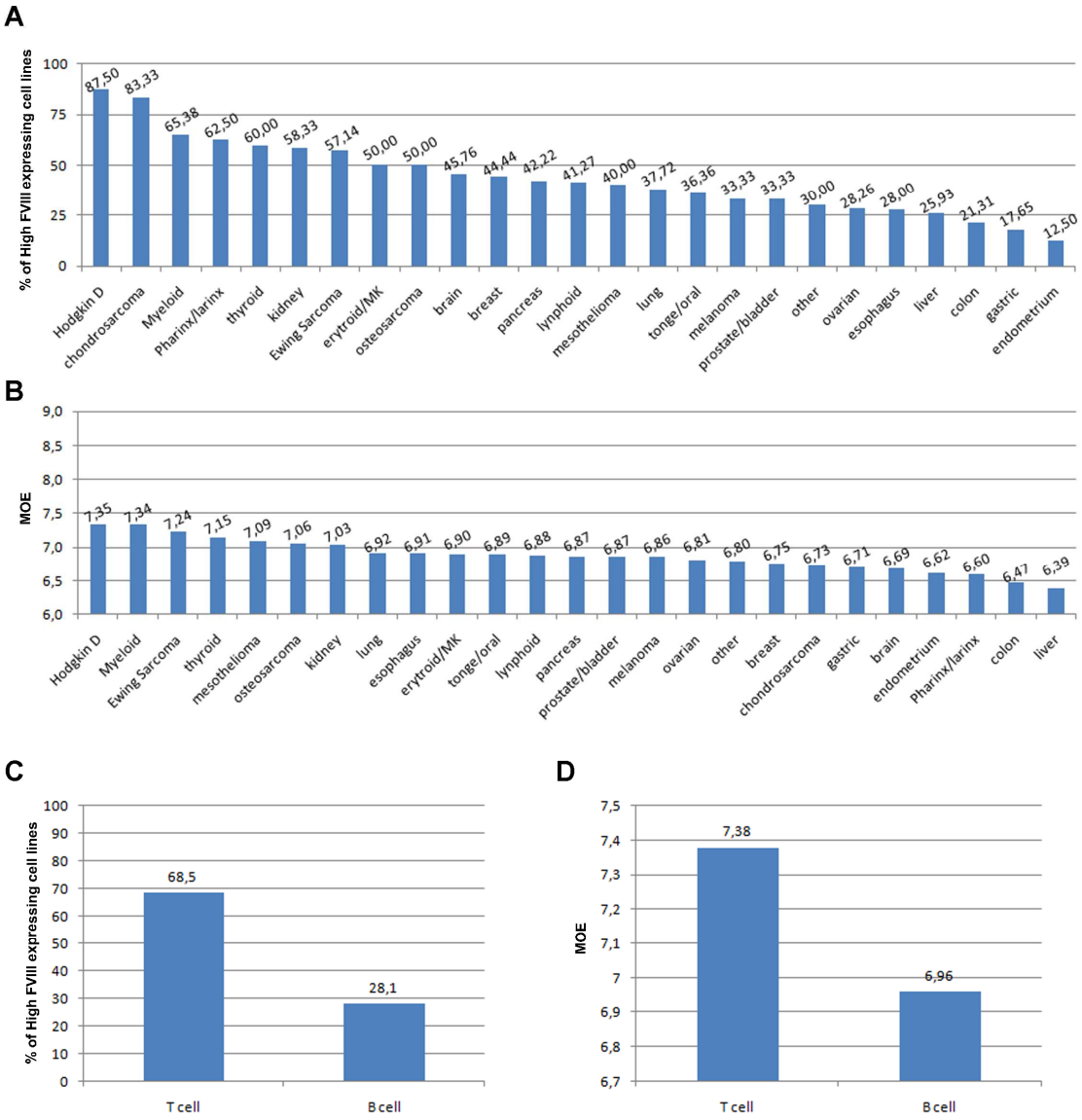

A
% of High FVIII expressing cell lines
100
75
50
25
0
87,50
83,33
65,38
62,50
60,00
58,33
57,14
50,00
50,00
45,76
44,44
42,22
41,27
40,00
37,72
36,36
33,33
33,33
30,00
28,26
28,00
25,93
21,31
17,65
12,50
Hodgkin D
chondrosarcoma
Myeloid
Pharinx/larinx
thyroid
kidney
Ewing Sarcoma
erytroid/MK
osteosarcoma
brain
breast
pancreas
lymphoid
mesothelioma
lung
tonge/oral
melanoma
prostate/bladder
other
ovarian
esophagus
liver
colon
gastric
endometrium
B
MOE
9,0
8,5
8,0
7,5
7,0
6,5
6,0
7,35
7,34
7,24
7,15
7,09
7,06
7,03
6,92
6,91
6,90
6,89
6,88
6,87
6,87
6,86
6,81
6,80
6,75
6,73
6,71
6,69
6,62
6,60
6,47
6,39
Hodgkin D
Myeloid
Ewing Sarcoma
thyroid
mesothelioma
osteosarcoma
kidney
lung
esophagus
erytroid/MK
tonge/oral
lymphoid
pancreas
prostate/bladder
melanoma
ovarian
other
breast
chondrosarcoma
gastric
brain
endometrium
Pharinx/larinx
colon
liver
C
% of High FVIII expressing cell lines
100
90
80
70
60
50
40
30
20
10
0
68,5
28,1
T cell
B cell
D
MOE
7,5
7,4
7,3
7,2
7,1
7
6,9
6,8
6,7
7,38
6,96
T cell
B cell

Fig.3

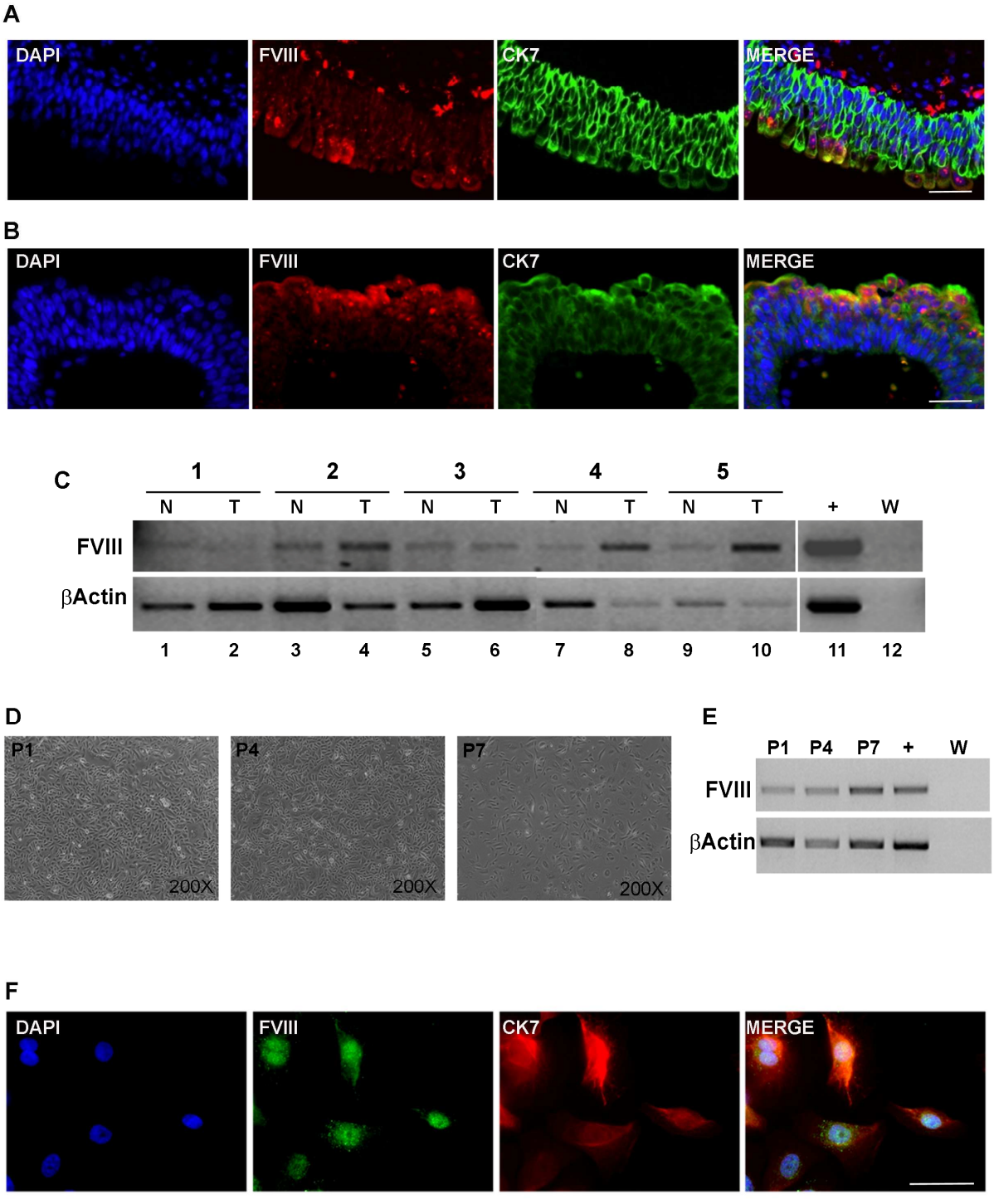

A
DAPI
FVIII
CK7
MERGE
B
DAPI
FVIII
CK7
MERGE
C
1
2
3
4
5
N
T
N
T
N
T
N
T
N
T
+
W
FVIII
βActin
1
2
3
4
5
6
7
8
9
10
11
12
D
P1
200X
P4
200X
P7
200X
E
P1
P4
P7
+
W
FVIII
βActin
F
DAPI
FVIII
CK7
MERGE

Fig.4

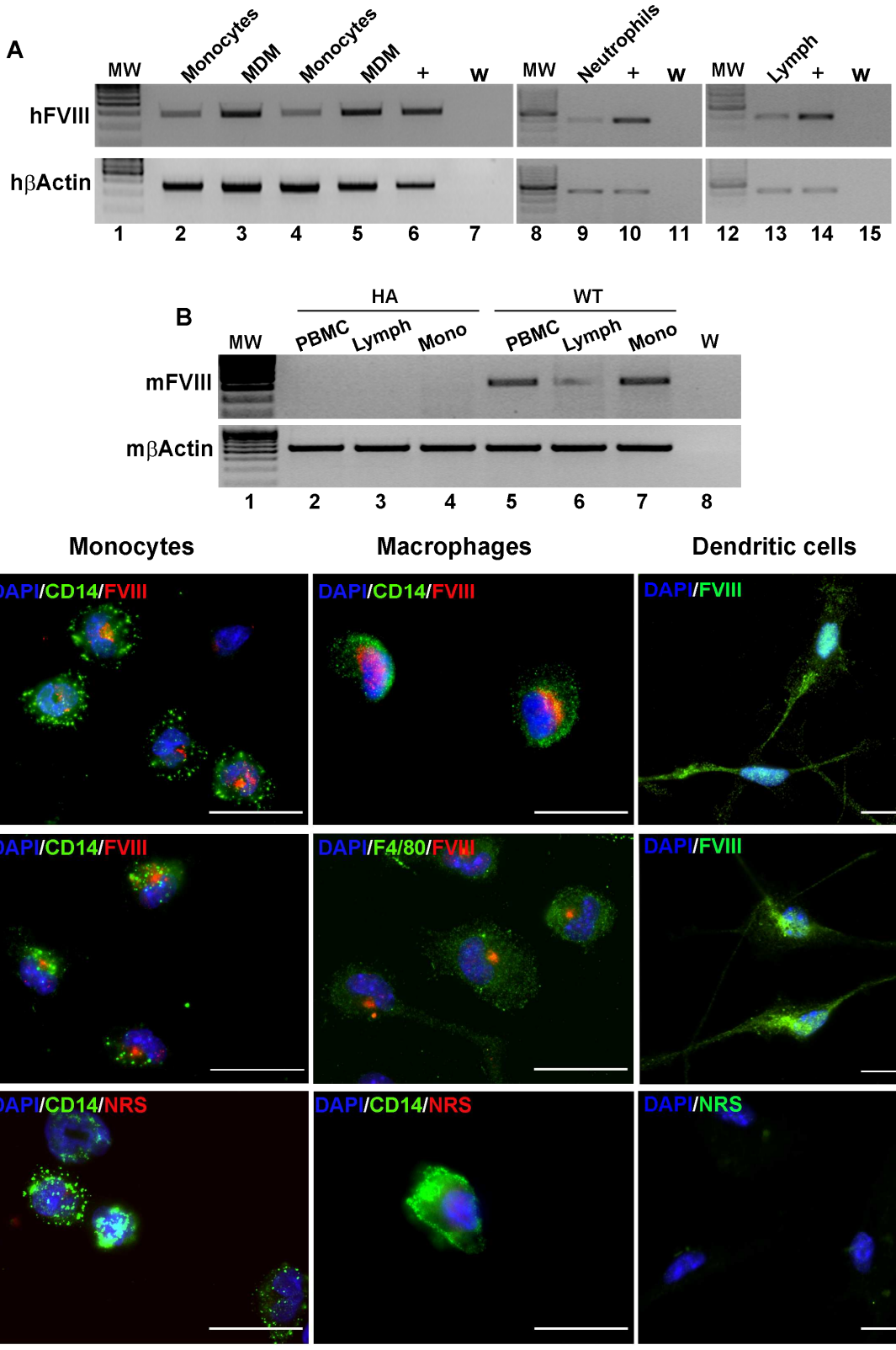

A
MW
Monocytes
MDM
Monocytes
MDM
+
w
MW
Neutrophils
+
w
MW
Lymph
+
w
hFVIII
hβActin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
HA
WT
MW
PBMC
Lymph
Mono
PBMC
Lymph
Mono
W
mFVIII
mβActin
1 2 3 4 5 6 7 8
Monocytes
Macrophages
Dendritic cells
C
Human
DAPI/CD14/FVIII
DAPI/CD14/FVIII
DAPI/FVIII
D
Mouse
DAPI/CD14/FVIII
DAPI/F4/80/FVIII
DAPI/FVIII
E
DAPI/CD14/NRS
DAPI/CD14/NRS
DAPI/NRS

Fig.5

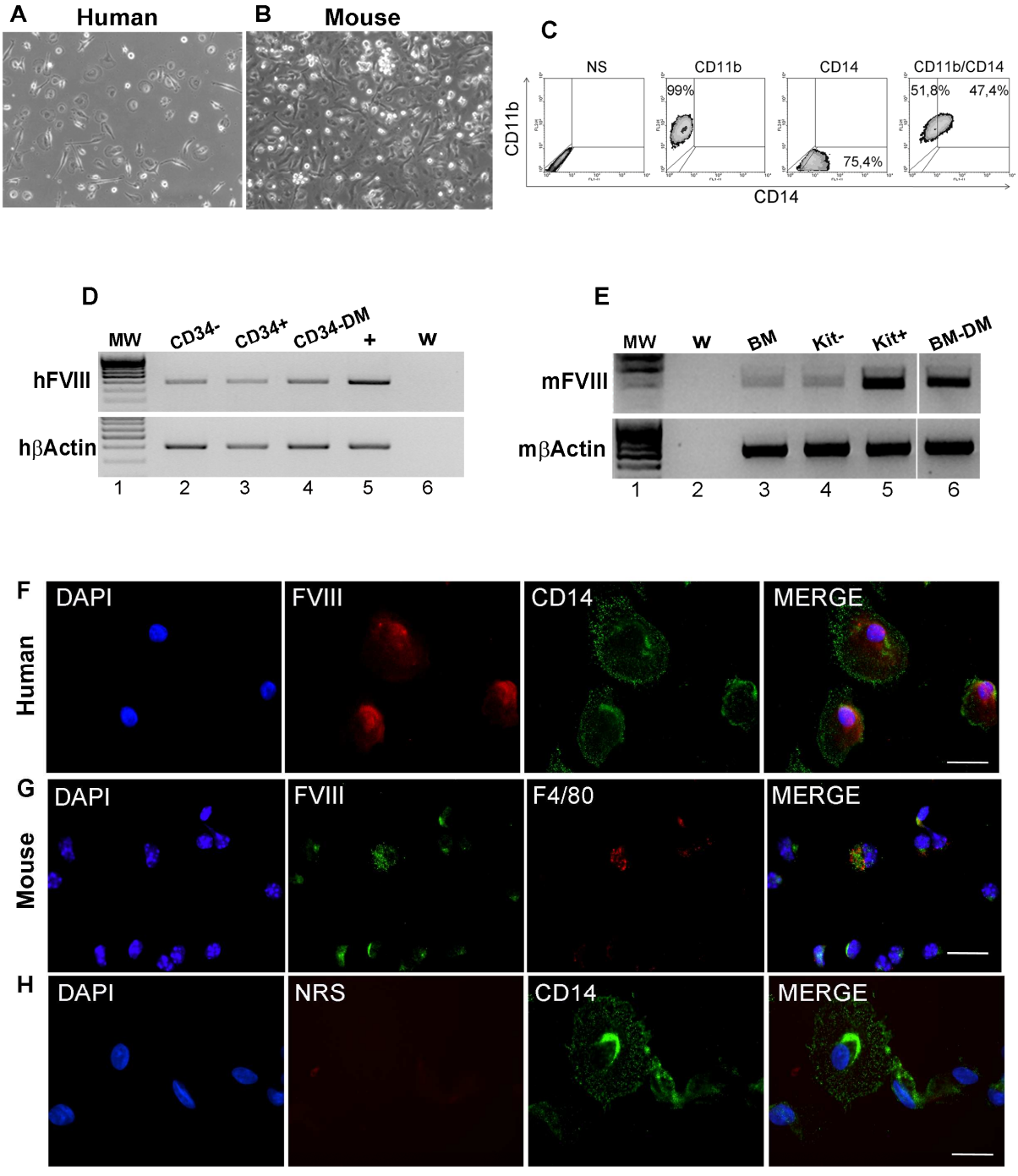

A
Human
B
Mouse
C
NS
CD11b
CD14
CD11b/CD14
99%
51,8%
47,4%
75,4%
CD11b
CD14
D
MW
CD34-
CD34+
CD34-DM
+
w
hFVIII
hβActin
1
2
3
4
5
6
E
MW
w
BM
Kit-
Kit+
BM-DM
mFVIII
mβActin
1
2
3
4
5
6
F
Human
DAPI
FVIII
CD14
MERGE
G
Mouse
DAPI
FVIII
F4/80
MERGE
H
DAPI
NRS
CD14
MERGE

Fig.6

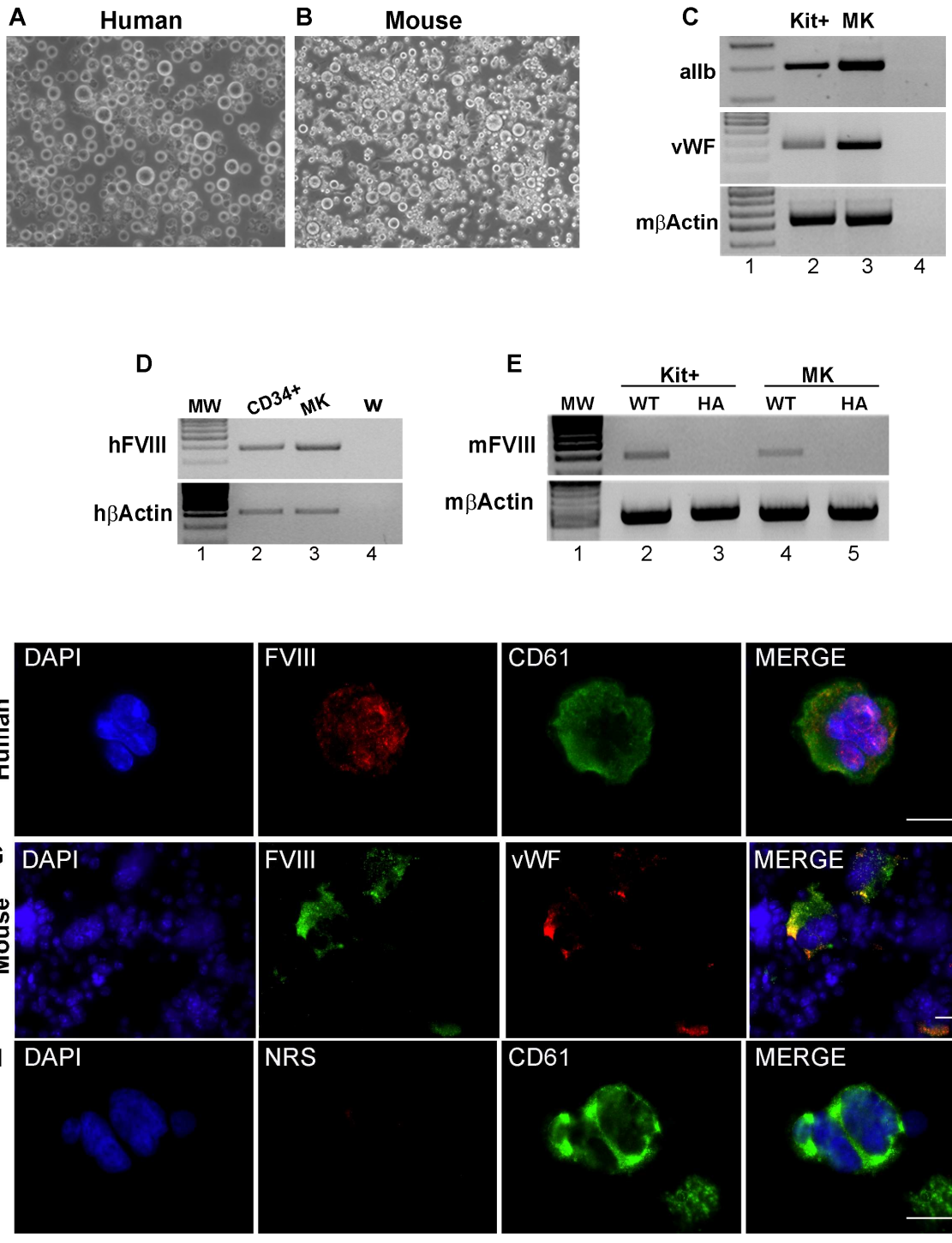

A
Human
B
Mouse
C
Kit+ MK
αIIb
vWF
mβActin
1 2 3 4
D
MW CD34+ MK W
hFVIII
hβActin
1 2 3 4
E
Kit+
MK
MW WT HA WT HA
mFVIII
mβActin
1 2 3 4 5
F
Human
DAPI
FVIII
CD61
MERGE
G
Mouse
DAPI
FVIII
vWF
MERGE
H
DAPI
NRS
CD61
MERGE

Fig. 7

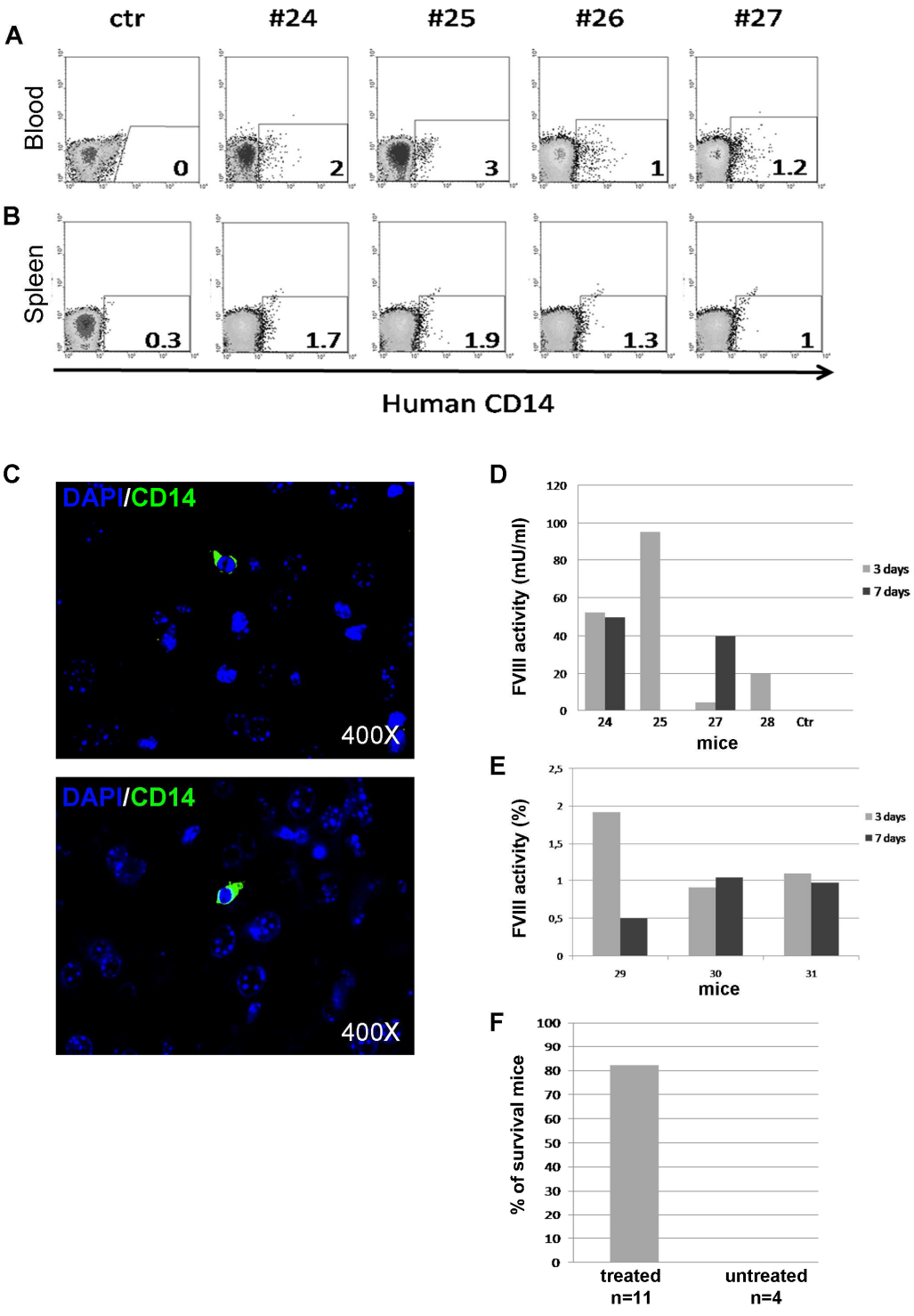

A
ctr
#24
#25
#26
#27
Blood
0
2
3
1
1.2
B
Spleen
0.3
1.7
1.9
1.3
1
Human CD14
C
DAPI/CD14
400X
DAPI/CD14
400X
D
FVIII activity (mU/ml)
120
100
80
60
40
20
0
24
25
27
28
Ctr
mice
3 days
7 days
E
FVIII activity (%)
2,5
2
1,5
1
0,5
0
29
30
31
mice
3 days
7 days
F
% of survival mice
100
90
80
70
60
50
40
30
20
10
0
treated
n=11
untreated
n=4

Fig.8

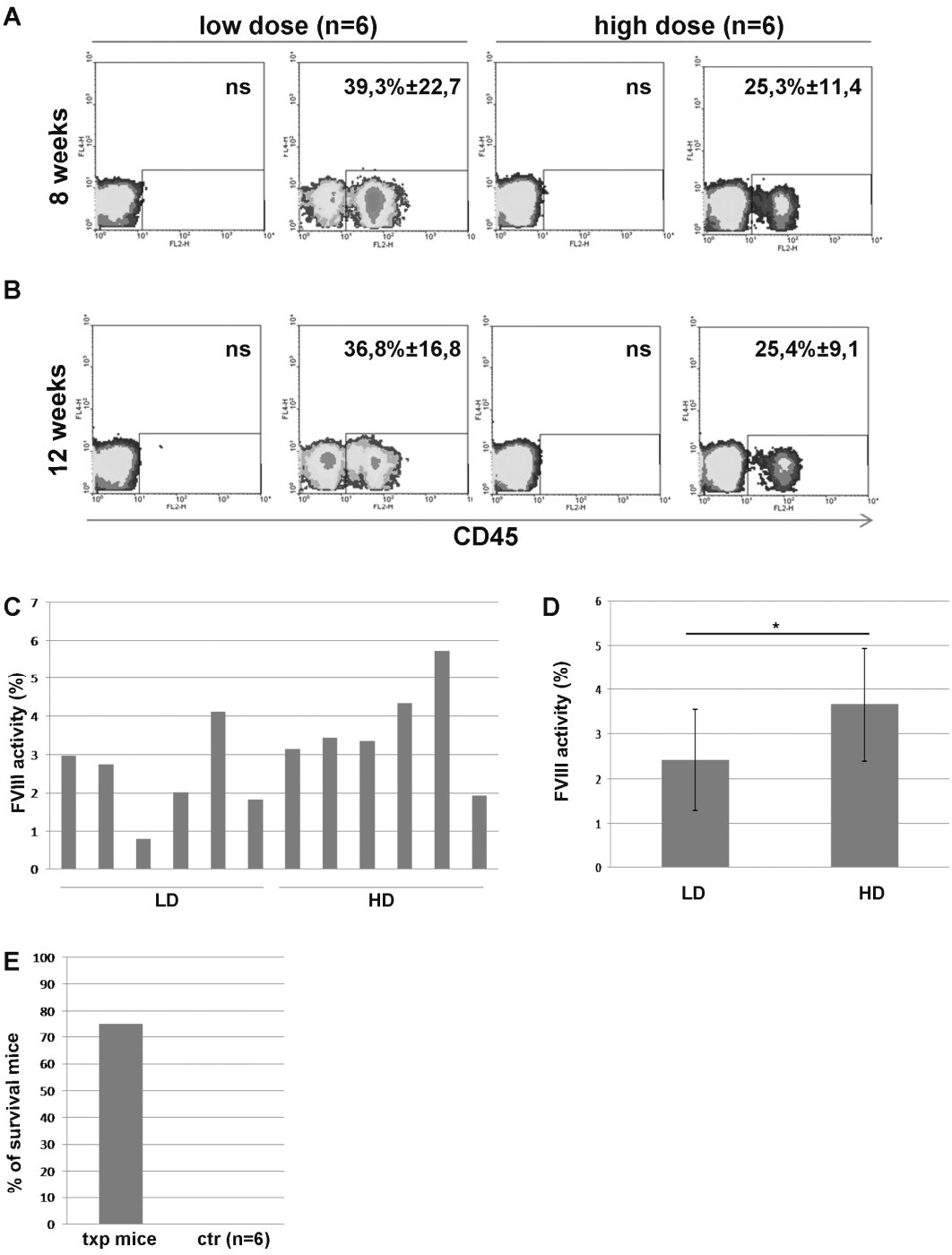

A
low dose (n=6)
high dose (n=6)
8 weeks
ns
39,3%±22,7
ns
25,3%±11,4
B
12 weeks
ns
36,8%±16,8
ns
25,4%±9,1
CD45
C
FVIII activity (%)
LD
HD
D
FVIII activity (%)
*
LD
HD
E
% of survival mice
txp mice
ctr (n=6)

Fig.9

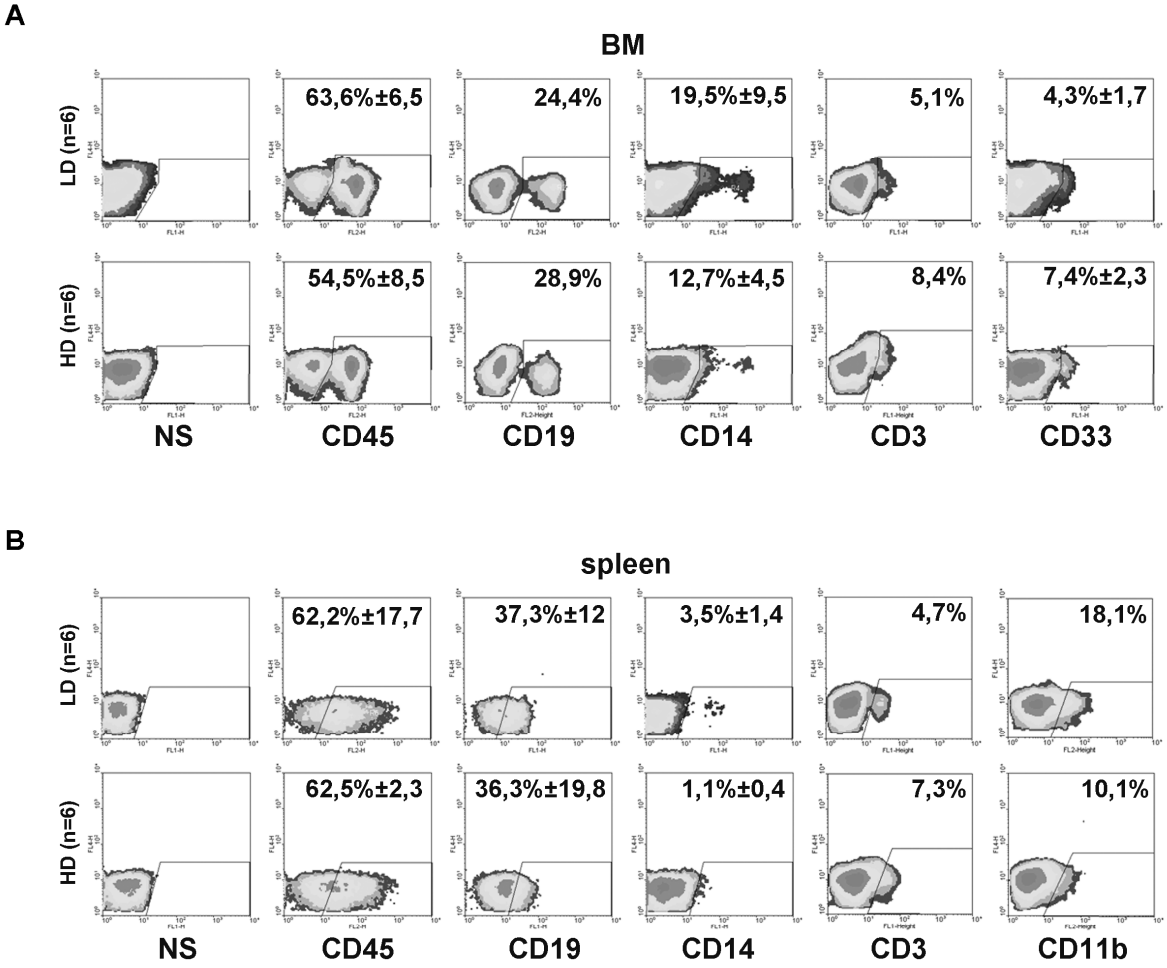

A
BM
LD (n=6)
HD (n=6)
63,6%±6,5
24,4%
19,5%±9,5
5,1%
4,3%±1,7
54,5%±8,5
28,9%
12,7%±4,5
8,4%
7,4%±2,3
NS
CD45
CD19
CD14
CD3
CD33
B
spleen
LD (n=6)
HD (n=6)
62,2%±17,7
37,3%±12
3,5%±1,4
4,7%
18,1%
62,5%±2,3
36,3%±19,8
1,1%±0,4
7,3%
10,1%
NS
CD45
CD19
CD14
CD3
CD11b

Fig.10

A

Transcriptional Factor	N of recognize sequence		Expression and function
	SpF8 (1175bp)	LpF8 (2300bp)	
TFII-D (7 nt)	5	5	RNA Pol II
TBP (10 nt)	3	3	TATA binding protein
HNF3-alfa (8 nt)	2	4	Hepatocytes
HNF1-alfa (8 nt)	3	5	Hepatocytes
C/EBP-alfa (7 nt)	5	8	Hepatocytes, mieloyd differentiation
c-Ets-1 (7 nt)	7	8	Endothelial cells
c-Ets-2 (9 nt)	4	4	Endothelial cells
PEA 3 (9 nt)	2	2	c-Ets family
STAT4 (6 nt)	16	22	Mieloyd lineage
GATA-1 (6 nt)	8	19	Mieloyd lineage
NF-Y (8 nt)	2	4	Increasing during monocytes-macrophages differentiation
IRF-2 (6 nt)	3	3	Monocytes
STAT1 (10 nt)	1	2	Hematopoietic cells
TCF-4E (10 nt)	1	1	B cells
Pax5 (7 nt)	3	4	B cells
NF-AT1 (10 nt)	2	4	T cells
Fox P3 (7 nt)	3	11	T regulatory cells
LEF-1 (8 nt)	0	2	Pre B pre T cells

B

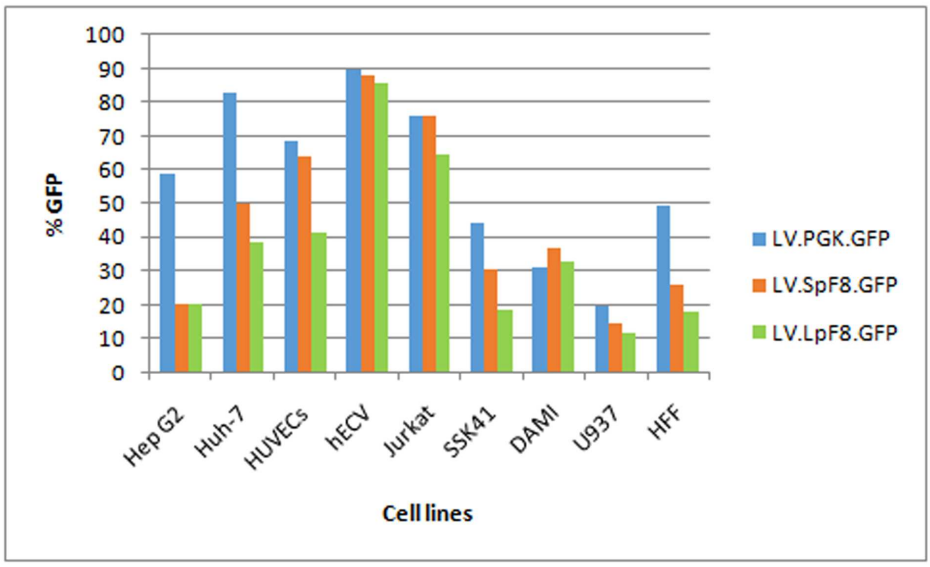


Fig.11

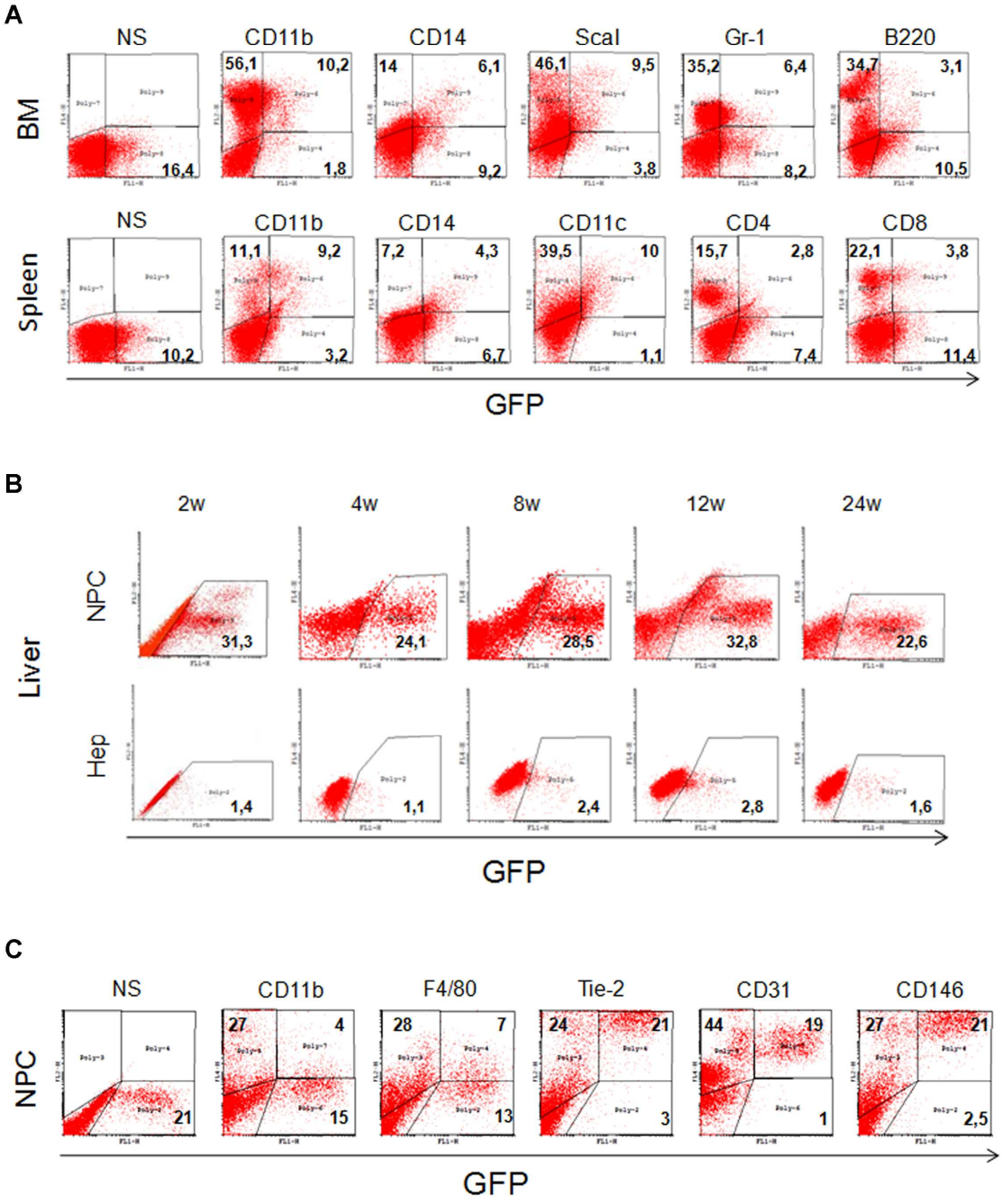

A
NS
CD11b
CD14
ScaI
Gr-1
B220
BM
56,1
10,2
1,8
14
6,1
9,2
46,1
9,5
3,8
35,2
6,4
8,2
34,7
3,1
10,5
16,4
NS
CD11b
CD14
CD11c
CD4
CD8
Spleen
11,1
9,2
3,2
7,2
4,3
6,7
39,5
10
1,1
15,7
2,8
7,4
22,1
3,8
11,4
10,2
GFP
B
2w
4w
8w
12w
24w
Liver
NPC
31,3
24,1
28,5
32,8
22,6
Hep
1,4
1,1
2,4
2,8
1,6
GFP
C
NS
CD11b
F4/80
Tie-2
CD31
CD146
NPC
27
4
15
28
7
13
24
21
3
44
19
1
27
21
2,5
21
GFP

Fig.12

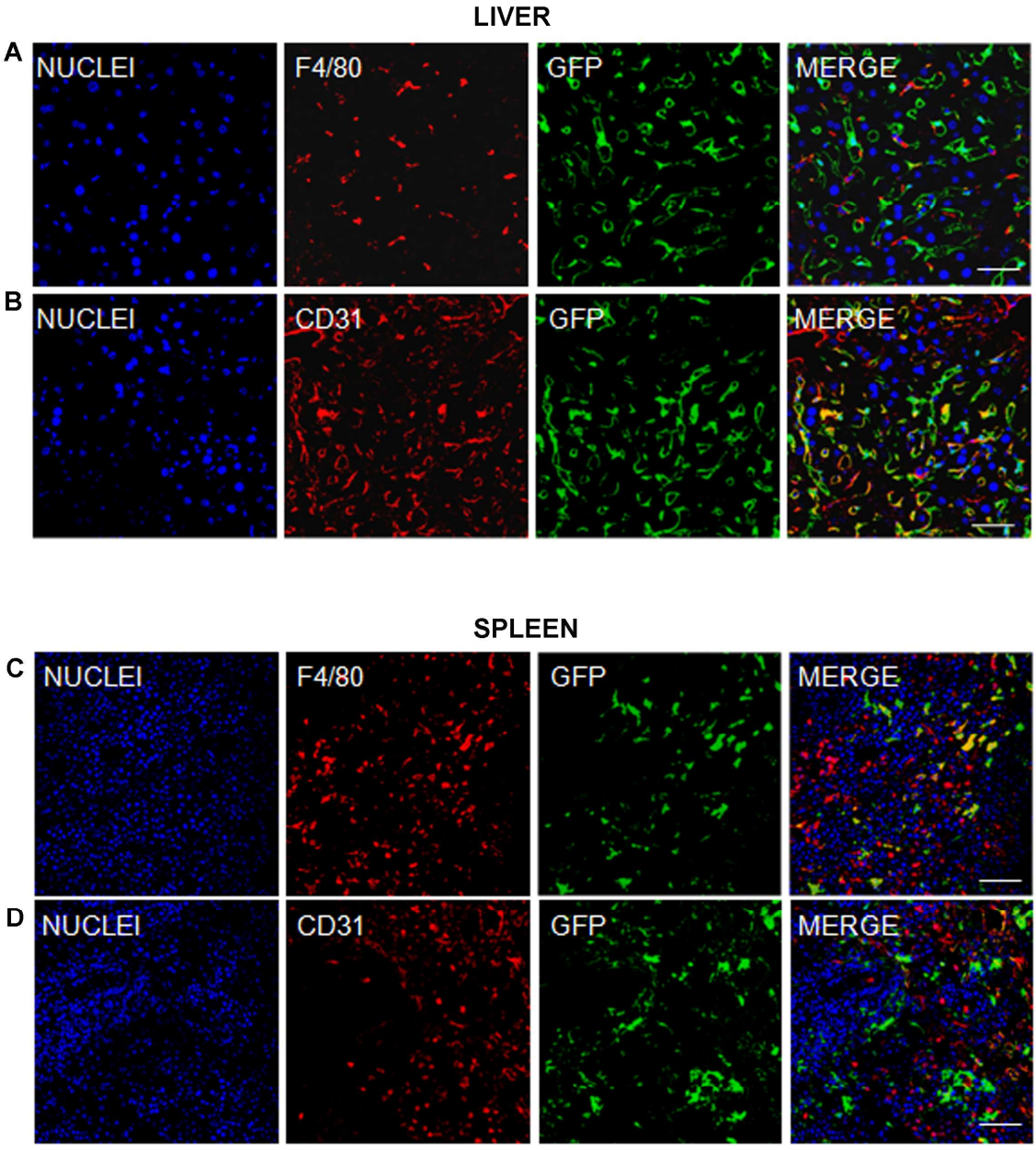

LIVER
A
NUCLEI
F4/80
GFP
MERGE
B
NUCLEI
CD31
GFP
MERGE
SPLEEN
C
NUCLEI
F4/80
GFP
MERGE
D
NUCLEI
CD31
GFP
MERGE

Fig.13

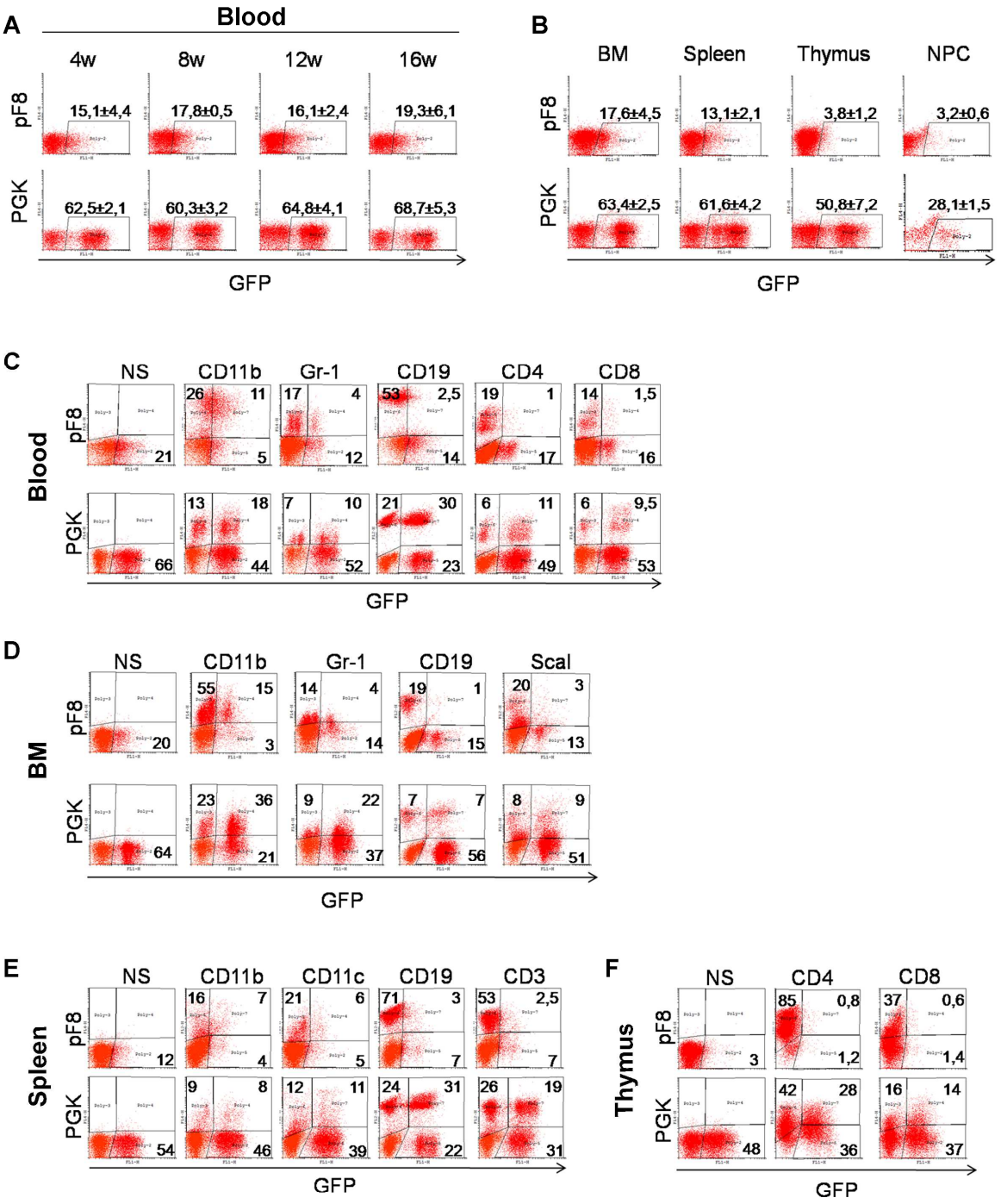

A
Blood
4w
8w
12w
16w
pF8
15,1±4,4
17,8±0,5
16,1±2,4
19,3±6,1
PGK
62,5±2,1
60,3±3,2
64,8±4,1
68,7±5,3
GFP
B
BM
Spleen
Thymus
NPC
pF8
17,6±4,5
13,1±2,1
3,8±1,2
3,2±0,6
PGK
63,4±2,5
61,6±4,2
50,8±7,2
28,1±1,5
GFP
C
Blood
NS
CD11b
Gr-1
CD19
CD4
CD8
pF8
26 11 21 5
17 4 12
53 2,5 14
19 1 17
14 1,5 16
PGK
13 18 66 44
7 10 52
21 30 23
6 11 49
6 9,5 53
GFP
D
BM
NS
CD11b
Gr-1
CD19
Scal
pF8
55 15 20 3
14 4 14
19 1 15
20 3 13
PGK
23 36 64 21
9 22 37
7 7 56
8 9 51
GFP
E
Spleen
NS
CD11b
CD11c
CD19
CD3
pF8
16 7 12 4
21 6 5
71 3 7
53 2,5 7
PGK
9 8 54 46
12 11 39
24 31 22
26 19 31
GFP
F
Thymus
NS
CD4
CD8
pF8
85 0,8 3 1,2
37 0,6 1,4
PGK
42 28 48 36
16 14 37
GFP

Fig.14

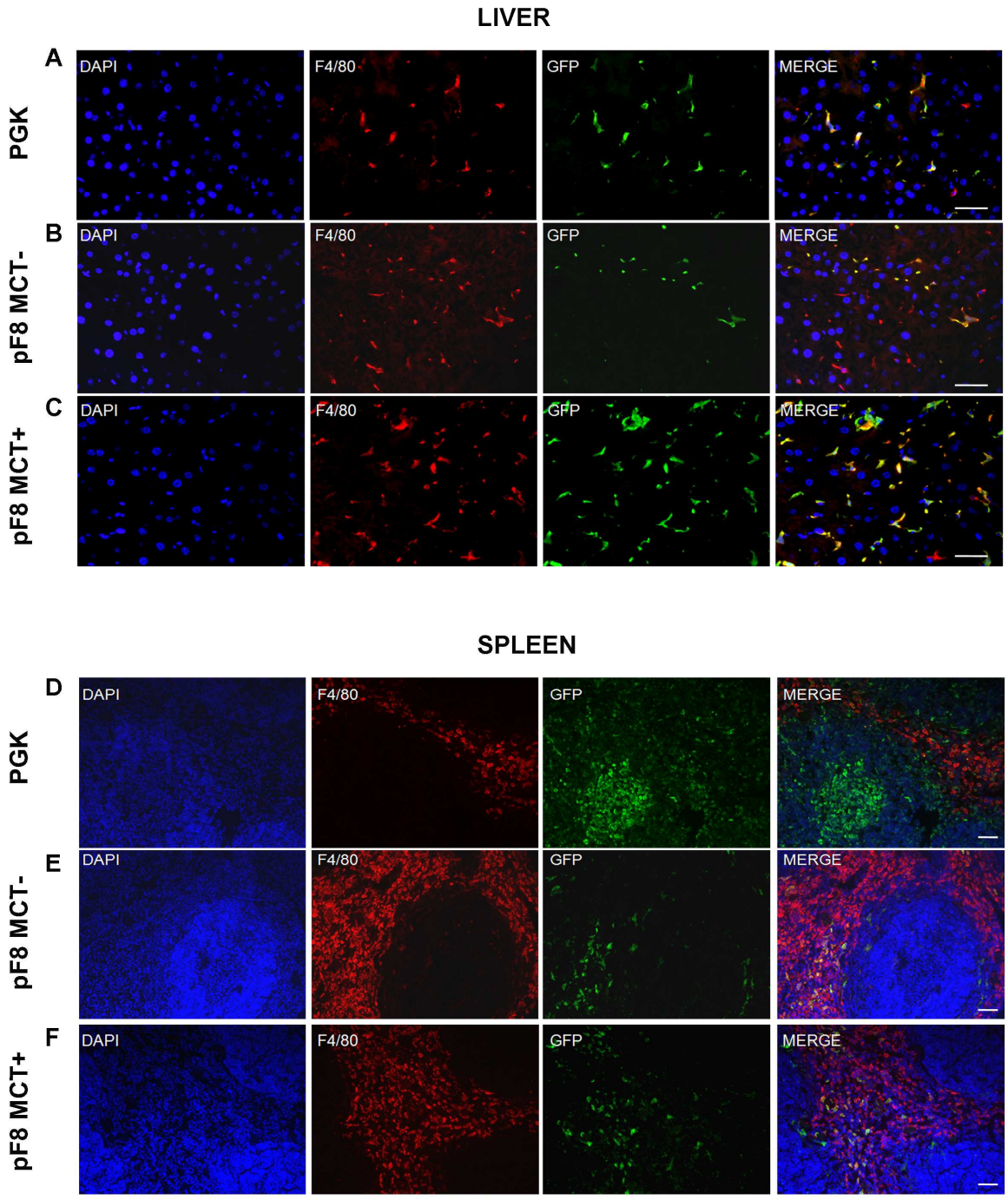
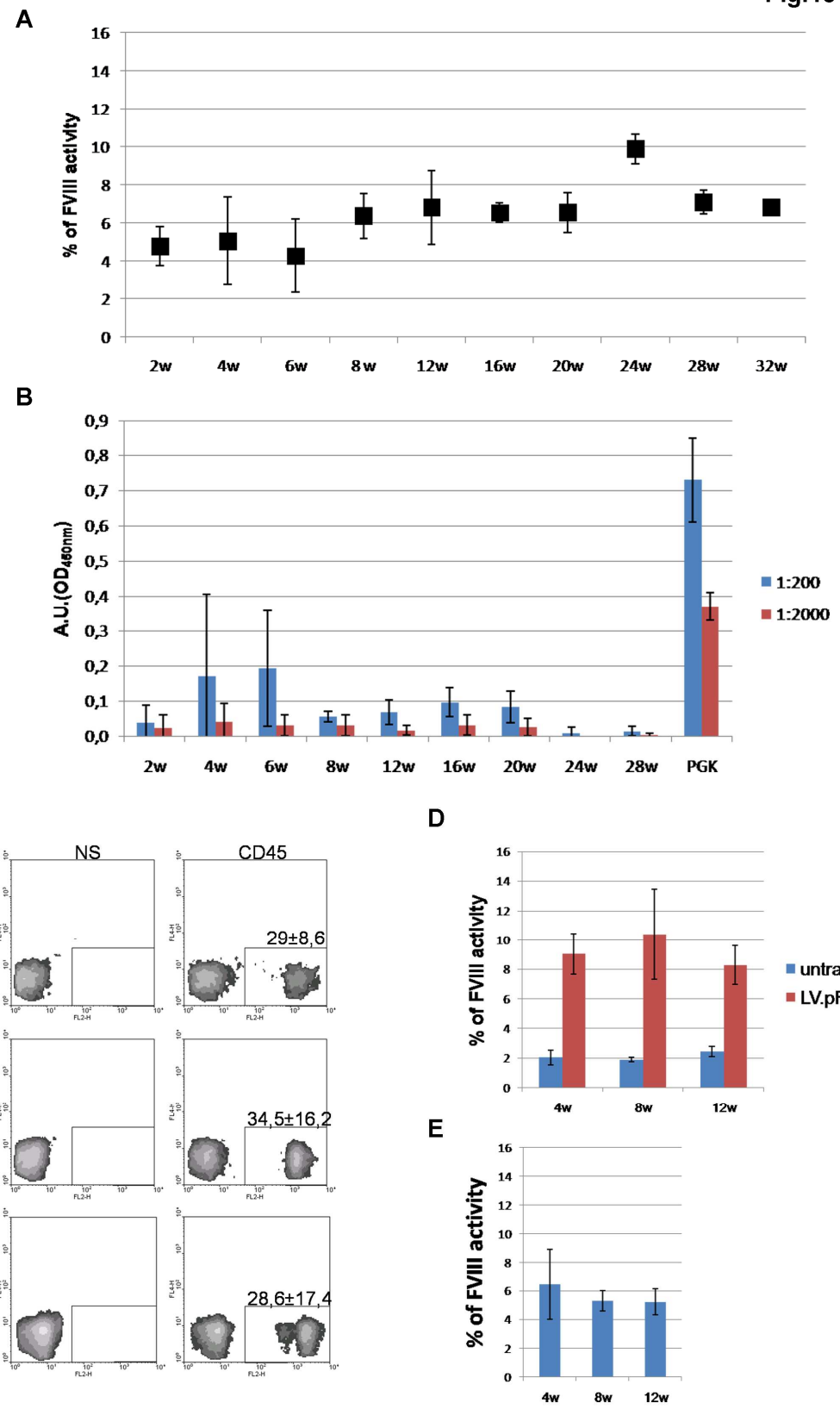

LIVER
A
PGK
DAPI
F4/80
GFP
MERGE
B
pF8 MCT-
DAPI
F4/80
GFP
MERGE
C
pF8 MCT+
DAPI
F4/80
GFP
MERGE
SPLEEN
D
PGK
DAPI
F4/80
GFP
MERGE
E
pF8 MCT-
DAPI
F4/80
GFP
MERGE
F
pF8 MCT+
DAPI
F4/80
GFP
MERGE

Fig.15


A
% of FVIII activity
16
14
12
10
8
6
4
2
0
2w
4w
6w
8w
12w
16w
20w
24w
28w
32w
B
A.U.(OD450nm)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
2w
4w
6w
8w
12w
16w
20w
24w
28w
PGK
1:200
1:2000
C
NS
CD45
4w
8w
12w
29±8,6
34,5±16,2
28,6±17,4
D
% of FVIII activity
16
14
12
10
8
6
4
2
0
4w
8w
12w
untransduced
LV.pF8.FVIII
E
% of FVIII activity
16
14
12
10
8
6
4
2
0
4w
8w
12w

Legend to figures

Fig.1: Expression of FVIII in human liver cells.

Human LSEC, KC and hepatocytes were isolated and FVIII expression was evaluated by immunofluorescence (A-E), and qPCR (G). Identity of isolated LSEC and KC was verified by FACS analysis (F). (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-2+ and CD31+). (G) qPCR showing FVIII mRNA expression in human liver and isolated human LSEC, KC and hepatocytes. NRS, normal rabbit serum. Scale bar: 25μm. *: p.Value <0,05

Fig.2: FVIII expression analysis in cancer cell lines.

FVIII expression data from transcriptome analysis of 909 cancer cell lines were analyzed by tumor clusterization. Each cluster were ordered according to the percentage of high expressing cell lines (A) and to the mean of expression (MOE) (B). Myeloid cluster showed high score of FVIII expression considering both parameters (A, B). Conversely, hepatocytes cluster displayed majority of cell lines with score lower than 7,1 and among the cluster, showed the lowest FVIII MOE value (A, B). (C, D) Further stratification of lymphoid cluster showed high FVIII expression in T cell compared to B cell.

Fig.3: FVIII expression in normal and bladder tissue and in primary bladder epithelial cells.

IF staining showed FVIII positivity in normal (A) and cancer (B) bladder tissue at epithelial level with increased signal in tumor. RT-PCR demonstrated FVIII expression in several bladder specimens with higher intensity in cancer tissue compared to normal (C). Bladder epithelial cell displayed typical epithelial morphology at different passages (D) and expressed FVIII at mRNA (E) and protein level (F). IF showed in cultured BEC co-expression between FVIII and CK7 (F). Scale bar: 25μm.

Fig.4: FVIII expression in peripheral blood derived cells.

RT-PCR analysis of the expression of FVIII in human monocytes isolated from peripheral blood, monocytes-derived macrophages (MDM), neutrophils, lymphocytes (lymph) (A) and in PBMC, monocytes and lymphocytes isolated from mouse blood (B). No FVIII signal was detected in cells isolated from HA mice (B). FVIII immunoreactivity on human (A) and mouse (B) monocytes, macrophages and dendritic cells. (E) Control staining using normal rabbit serum (NRS) Scale bar: 25μm.

Fig.5: FVIII expression in hematopoietic stem cell derived macrophages.

Morphology at contrast phase microscopy of human CD34+ cells and mouse BM cells differentiated in macrophages with a cocktail of cytokines (A, B). Cytofluorimetric analysis of the differentiated human macrophages, which expressed the expected markers CD11b and CD14 (C). RT-PCR on CD34 (D) and mouse BM (E) derived macrophages show FVIII expression in these cells. FVIII protein is detected by immunofluorescence in both in human and mouse HSC derived macrophages (F, G). CD34-DM, CD34 derived macrophages. Scale bar: 25μm.

Fig.6: FVIII expression in hematopoietic stem cell derived megakaryocytes.

Morphology at contrast phase microscopy of human CD34+ cells and mouse Kit+ cells differentiated in MK with a cocktail of cytokines (A, B). RT-PCR analysis of mouse Kit+ cells and MK for aIIb and vWF specific markers (C). RT-PCR on CD34+ (D) and mouse Kit+ (E) derived MK show FVIII expression in these cells. FVIII protein is detected by immunofluorescence in both in human and mouse HSC derived MK (F,G). Scale bar: 25μm.

Fig. 7 Hemophilia A correction after monocyte injection in hemophilic mice.

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

Fig.8: FVIII activity in hCD34+ transplanted mice.

Transplanted human cells were identified in the mouse blood by cytofluorimetry for human CD45 marker. Human chimerism was assessed 8 and 12 weeks after CD34 transplantation (A, B). FVIII activity of transplanted mice was assessed 3 months after hCD34+ injection by aPTT. All treated mice showed FVIII activity (C) with levels between 2 and 5% (D). Three months after transplantation mice were challenged with tail clip assay and 9 out 12 (75%) survived (E). ns, not stained; LD, low dose; HD, high dose. *: p.Value <0,05

Fig.9: Human chimerism in BM and spleen of transplanted mice.

BM and spleens of transplanted mice were stained for human markers 3 months after transplantation. BM was stained for hCD45, hCD33 myeloid precursor, hCD14 monocytes marker, hCD3 T cell marker and hCD19 B cell marker (A). Meanwhile spleen was analyzed for the presence of hCD45, hCD14, CD19, hCD3 and CD11b monocytes marker (B). Percentages without standard deviations are representative of two independent experiments. NS, no stained; LD, low dose; HD, high dose.

Fig.10: *In silico* and *in vitro* analysis of long (LpF8) and short (SpF8) FVIII promoter sequences.

In silico analysis of transcriptional factor consensus sequences on SpF8 and LsPF8, predicted the presence of several hematopoietic-specific TF (green box), in addition to hepatocytes- (bleu box) and endothelial-specific TF (red box). In parentheses near the TF name are reported the numbers of nucleotide recognized (A). To evaluate pF8 activity in vitro several cell line were transduced with LV.SpF8 and LV.LpF8. Both promoters were less active in hepatocytes, and human foreskin fibroblast (HFF) if compared with GFP expression driven by the ubiquitous promoters PGK.

Fig.11: FVIII promoter activity *in vivo*.

To analyzed pF8 activity, *in vivo* several mice were injected with LV.SpF8.GFP. FACS analysis on BM and spleen (1 month after LV injection) showed that pF8 is mainly active in myeloid cells as shown by CD11b, CD11c, CD14 and Gr-1 co-staining. Few positivity was observed in lymphoid cells stained for B220, CD4 and CD8 (A). In the liver GFP expression was restricted over time to non-parenchymal cells (NPC) meanwhile hepatocytes were barely detected (B). In the NPC GFP+ cells are predominantly endothelial and less macrophages (C).

Fig.12: FVIII promoter drive GFP expression differentially in liver and spleen.

Representative immunofluorescences of mouse liver and spleen 8 weeks after LV.SpF8.GFP injection. In the liver FVIII promoter is predominantly active in LSEC as shown by co-staining between GFP and CD31 endothelial marker (A). Few KC stained for F4/80 marker expressed the GFP (B). IF on spleen showed GFP signal mainly in F4/80 macrophages (C) and low co-staining was observed between GFP and CD31 (D). Scale bar=25μm

Fig.13: GFP distribution in mice after SpF8-Lin- transplantation

FACS analysis showed presence of GFP positive cells higher than 16% on blood of SpF8-Lin- transplanted mice over time (A). (B) GFP expression in BM, Spleen, Thymus and liver NPC of treated mice 4 months after transplantation. FACS analysis of blood (C), BM (D) and spleen (E) of lin-pF8 transplanted mice showed most of GFP expressing cells positive for myeloid markers in comparison with lymphoid. In thymus pF8 drive low GFP expression in CD4 and CD8 positive cells.

Fig.14: GFP expression in liver and spleen of SpF8-Lin- transplanted mice and after MCT treatment

IF showed GFP-F4/80 double positive cells in SpF8-Lin- and PGK-Lin- transplanted mice liver (A,B). Increase of GFP expressing KC was observed in the after MCT treatment of SpF8-Lin- mice (C). IF staining of spleen showed GFP expression restricted to F4/80 positive cells in SpF8-Lin- mice (E). (D) PGK extend GFP expression in germinative center cells other than in F4/80 splenic macrophages. (F) No differences occurs in spleen after MCT treatment of SpF8-Lin- mice.

Figure 15. *In vivo* and *ex vivo* gene therapy using LV.pF8.FVIII

aPTT assay showed sustained FVIII activity on plasma of hemophilia A mice up to 32 weeks after LV.SpF8.FVIII injection (A). aPTT. (B) By ELISA, very low levels of anti-FVIII antibodies were detected after LV.SpF8.FVIII injection in comparison with LV.PGK.FVIII. 1:200 and 1:2000 indicate the dilution of plasma used in ELISA. (C) FACS for human CD45 showed human chimerism in pF8.FVII-CD34+ transplanted NSG-HA mice up to 3 months month after transplant. aPTT assay showed therapeutic levels of FVIII after transplantation of LV.pF8.FVIII modified CD34 (D) or Lin- cells (E).

Discussion

The current therapy used in hemophilia A patients is the parenteral administration of blood products or recombinant FVIII. However, this solution do not represent a definitive cure and is associated with some disadvantages such as the high costs due to the frequent delivery of FVIII requested in the prophylaxis protocol and the risk of developing neutralizing antibodies against FVIII occurring in 30-40% of patients with the severe form (9). For this reason, other approaches able to provide continuous therapeutic efficacy and minimize the side effects of replacement therapy are strongly needed. It was recently introduced the use of recombinant FVIII molecules directly conjugated to polyethylene glycol (PEG) or linked to the neonatal FcR with the dual purpose to increase the FVIII half-life in circulation and reduce the antibodies recognition (198). Despite some of these improved molecules extend FVIII half-life and reached phase three clinical trial, they did not provide a significant reduction in number of administration so far (24). Besides the molecular protein optimization for replacement therapy, cell and gene therapy could also represent an interesting and promising resource for the medical treatment of the disease. In cell therapy perspective, it is essential to identify the cell source that is capable to produce FVIII and, at the same time, is able to meet the necessary conditions to ensure the transplant success. At this point liver is the main organ producing FVIII in the body. Among liver cell types, LSEC were recently reported to be the unique source of FVIII (77-79), although, several authors, even in early years, described both mRNA and protein presence also in hepatocytes (69-72). In this study we analyzed FVIII expression in isolated human liver cells detecting FVIII in LSEC, hepatocytes and also in KC by qPCR and IF which have demonstrated that not only LSEC are FVIII producing cells in the liver. Nevertheless, LSEC showed higher FVIII signal in comparison to KC and hepatocytes confirming their major role in FVIII synthesis. Another important point of discussion is whether FVIII expressing cells are also able to release the factor, considering its complex secretion pathway. Several studies demonstrated that LSEC transplantation from healthy donors was able to correct the bleeding phenotypes of hemophilic mice (76, 82, 84), conversely more controversial results were obtained with hepatocytes (84, 85). Interestingly, we observed presence of FVIII protein and mRNA in human KC confirming our previous results reporting that also murine KC transplant increased survival in HA mice after tail clip assay in short term experiments (93). Taken together all this data demonstrate the importance of liver cells, mainly LSEC, in FVIII production and secretion. However, several authors have described FVIII expression also in other organs such as spleen, lungs, kidneys and in BM derived cells (80, 89, 93-95). Extrahepatic sources of FVIII was suggested earlier by Webster demonstrating that in dog transplantation of HA liver in normal recipient did not produced the disease (86). Among the

other organs, spleen transplantation showed to increase FVIII levels both in dogs and humans (89-92), however the cell types of extrahepatic sources involved in FVIII secretion were not characterized. Similarly to liver, endothelial cells of other organs were found to express FVIII (78) although the ability of other cells in this function cannot be excluded. Indeed, FVIII positivity was also detected in spleen and lung macrophages (75). This well correlates with our transcriptome analysis on FVIII expressing cell lines demonstrating that myeloid cluster showed high score of FVIII expression in term of number of cell lines overexpressing FVIII and mean of expression. On the other hand, hepatocarcinoma cell cluster showed low FVIII expression values confirming our data on weak FVIII production in hepatocytes. Among clusters, kidney cells is also situated in high expressing cell lines, with a score lower than myeloid cells. This is interesting because FVIII expression in kidneys glomeruli and in tubular epithelial cells was previously reported (94, 95). Moreover, by further stratification of the lymphoid cluster we observed high FVIII expression in T cells compared to B cells. The observation that hematopoietic cells express significant levels of FVIII is consistent with our previous report demonstrating that total BM transplantation from healthy donor to hemophilic mice was able to restore therapeutic levels of FVIII and correcting the bleeding phenotypes (93). This finding opens to new opportunities for hemophilia A treatment by the identification of a suitable FVIII source to employ in cell therapy.

Starting from this result, here we focused our attention on which hematopoietic cells are involved in FVIII synthesis and secretion. We demonstrated FVIII expression in several isolated and differentiated murine and human hematopoietic cells by both RT-PCR and IF. In particular, we detected higher expression in peripheral blood monocytes and monocytes derived cells such as macrophages and dendritic cells. Interestingly, FVIII expression increased during monocytes-macrophages differentiation suggesting that a positive transcriptional regulation occurred. In contrast with monocytes, a faint FVIII mRNA signal was assessed in human granulocytes and in mouse and human lymphocytes. Similarly to monocytes-derived-macrophages FVIII positivity was also observed in macrophages directly differentiated from HSC isolated from mouse BM or human CB. In view of the importance of vWF in FVIII function we analyzed megakaryocytes, which together with endothelial cells represent the main vWF producer in the body. In particular, in endothelial cells it has been showed that the interaction between FVIII and vWF occurs into the Weibel-Palade bodies before secretion and is crucial for FVIII activity. We detected FVIII in both human and mouse MK, and with latter well co-localized with vWF by immunofluorescence stainings. The presence of FVIII protein within the cells examined could be explained by their

ability to up take and storing FVIII from exogenous source. However, the prolonged maintenance in culture and the presence of mRNA strongly support the endogenous origin of detected FVIII protein. Nevertheless, as mentioned above FVIII expression does not necessarily means secretion so it is crucial to determine if hematopoietic cells are able to release significant amount of functional protein. Human cord blood CD11b+ cells transplantation in NOD-SCID HA mice showed the presence of circulating FVIII protecting mice from tail clip challenging up to 1 week after injection. However, only low FVIII activity (1%) was achieved in treated mice, probably due to the numbers of cells engrafted. For this reason HSC xenotransplantation was performed to assess whether hematopoietic cells after human chimerism achievement were able to restore therapeutic FVIII activity in NSG-HA mice in long term experiments. By this approach aPTT assay performed 3 months after transplant showed FVIII levels higher than 2% in all treated mice sufficient to allow survival in 9 out 12 animals after tail clip. FACS analysis of human CD45 marker in spleen and BM of transplanted mice demonstrated chimerism superior than 60% with major B cell commitment in comparison to myeloid and T cell differentiation. The impaired capability to give rise to all complete hematopoietic populations after xenotransplant was already reported in literature (199). Since we found that myeloid compartment is the main FVIII producer among the hematopoietic population, this commitment unbalance could explain the difference in FVIII activity observed in this study compared with our previously results transplanting murine BM in singenic mice in which FVIII levels were restored up to 20%. During a survey on human organs expressing FVIII we interestingly found a strong positivity in bladder with an increased FVIII signal in cancer tissues compared to normal by RT-PCR and immunofluorescence. Moreover as shown by co-staining with CK7, FVIII was predominantly expressed in epithelial cells, both in tissue context and after cell isolation and culture. For instance, the significance of this expression is to be elucidate but is possible that is related with the role of FVIII in molecular processes different from coagulation. Indeed, some evidences suggested a role of FVIII in bone formation and remodeling, showing that HA mice are affected from decreased bone mass and strength (200, 201). Moreover, some studies have also reported increased levels of FVIII antigen and activity in cancer patients, which correlates with an increased risk of thrombotic events (202-204). These studies, which were mainly observational, do not speculate on the biological mechanism underlying this increased secretion and did not discussed about the cell source that may be involved in the FVIII production. Since identity of cells producing FVIII in the body is still a topic of discussion and other cell types may be involved in its expression, the study of F8 promoter could offer a possibility to better understand this issue. A 1175 bp promoter region

before the ATG was characterized by Figueiredo in 1995 demonstrating the presence of four hepatocyte specific transcriptional factors able to bind this sequence (98). Luciferase reporter assay showed F8 promoter activity in hepatic cell lines, however the ability of this region to drive trangene expression in other cell lines or in vivo has not been investigated by the authors. Moreover, the identification of hepatic transcriptional factors was performed only on 3 out 20 TF binding sequences predicted by DNase I footprinting analysis (98). For these reasons a furher characterization on F8 promoter is needed to extent our knowledge on regulation of FVIII expression. First, we blast the 1175bp region (SpF8) and one more long sequence of 2350bp (LpF8) in a software for TF binding site prediction. As confirmation, we found the same hepatic TF identified by Figueiredo suggesting the bona fide of our prediction results. In addition, *in silico* analysis showed the presence of specific endothelial and hematopoietic, mainly myeloid, TF. This founding are in agreement with data about FVIII expression in endothelium and with our results supporting the evidence of FVIII production also in hematopoietic cells (93). A further confirmation derived from *in vitro* validation of SpF8 and LpF8 activity after several human cell lines transduction with a LV carrying GFP under the transcriptional regulation of these promoters. Indeed, both promoters were able to drive GFP expression in hematopoietic and endothelial cell lines. Interestingly, despite the prediction of hepatocytes TF, but according to our transcriptome analysis and qPCR on human liver cells, F8 promoters were less active to induce transgene expression in hepatocarcinoma cell lines. However *in silico* and *in vitro* analysis did not showed significative differences between SpF8 and LpF8. For *in vivo* experiments several mice were tail vein injected with LV.SpF8.GFP in order to evaluate in which cells and organs F8 promoter was active. In hematopoietic organs such as spleen and BM, GFP expression was restricted in myeloid cells whereas low positivity was observed in lymphocytes. On the other hand in the liver F8 promoter was active mainly in NPC meanwhile GFP positive hepatocytes were rarely detected. In particular, by FACS and IF GFP expression in the liver was sustained predominantly in LSEC overtime. Moreover we observed an interestingly pattern of expression between liver and spleen. Indeed, in the liver F8 promoter was active mainly in endothelial cells, while in the spleen GFP expression was mostly restricted in F4/80 positive macrophages. Focusing on pF8 specificity in hematopoietic cells, LV.pF8.GFP transduced Lin- transplantation demonstrated promoter activity in blood cells and in hematopoietic cells engrafted in several organs such as spleen, thymus and liver other than BM. In addition, FACS analysis of blood, BM and spleen clearly indicated that pF8 was mainly active in myeloid cells whereas few GFP positivity was detected in lymphocytes. In the liver we detected F4/80 KC expressing GFP by IF,

moreover the number of these double positive cells greatly increased 10 days after MCT injection in mice, suggesting a positive regulation of pF8 in monocytes-macrophages during an inflammatory process. As confirmation of FACS analysis, IF in the spleen of transplanted mice showed GFP expression mainly in macrophages and in contrast with PGK-Lin- transplanted mice, no GFP was detected in splenic germinative centres, supporting the hypothesis that F8 promoter is specifically active in myeloid cells rather than in lymphocytes. Taken together *in silico*, *in vitro* and *in vivo* analysis strongly demonstrated that F8 promoter is active in endothelial cells and also in hematopoietic cells with higher specificity in myeloid population. Indeed, other than the well-known contribution of LSEC in FVIII production, by these experiments, we showed that in organs different from liver FVIII is expressed also in other cells and particularly we assessed pF8 activity in spleen macrophages.

Despite the success in hemophilia B using AAV (154), FVIII is associated with major drawbacks that impaired gene therapy. Above all, the main issue is related to inhibitors formation due to FVIII immunogenicity. It has demonstrated that driving FIX expression specifically in hepatocytes was sufficient to reduce antibodies formation and improving outcomes of gene therapy (140, 141). However, FIX is 5-6 time less immunogenic than FVIII and hepatocytes targeting did not definitively solved the inhibitors problem (128). Best results were obtained by avoiding off-target expression in APC using the mirT-142 in combination with an hepatocytes restricted targeting (173). Moreover FVIII gene therapy using a MK specific promoter allowed to reach therapeutic levels of FVIII even in presence of pre-existing inhibitors (180). However, other cell-specific targeting could be investigated in gene therapy application to achieve therapeutic FVIII levels and induce immunotolerance. The long-term GFP expression obtained using pF8, led us to investigate whether *in vivo* and *ex vivo* gene therapy using this promoter was able to induce FVIII expression able to ameliorate the clinical phenotype in HA mice. Tail vein injection of LV.pF8.FVIII in HA mice resulted in prolonged FVIII activity higher than 6% up to 8 months. Furthermore, this sustained expression well correlates with the absence of inhibitors formation, suggesting that cell targeting achieved by pF8 positively modulate the immunotolerance. At this point in our lab we obtained similar results by injecting HA with LV carrying FVIII under the control of a endothelial or myeloid specific promoter. These results further explains the good performance of pF8 since GFP studies have demonstrated its specificity for LSEC and myeloid cells. Also *ex vivo* gene therapy using LV.pF8.FVIII to transduce both murine and human HSC showed to increase FVIII levels up to 10% in HA mice after transplant. In particular, higher FVIII activity was achieved by transplanting human CD34+ in comparison

to murine Lin- and this difference could be explained by the fact that LV are more prone to transduce human cells and that, because of human origin, pF8 works with more efficiency in CD34+ derived transplanted cells. These studies demonstrated that pF8 is a suitable promoter for hemophilia A gene therapy by its ability to reach therapeutic FVIII levels and to prevent inhibitors formation. However, the immunological issue does not represent the only obstacle for HA gene therapy, indeed compared to other proteins of similar size, recombinant hFVIII expression is highly inefficient (189). Moreover, it is important to consider that scaling up to larger animals, with the final goal to achieve gene therapy in humans, a more rigorous evaluation on vector doses is required. At this point, to increase FVIII activity and possibly to reduce administration dose several codon optimized FVIII molecules were developed (30, 165, 189-192). The principle underlying many FVIII improvements consisted to ameliorate the FVIII secretion pathway by modifying the minimal B domain maintained in the BDD-FVIII. By this strategy highly active FVIII molecules were generated such as the FVIIIN6 and FVIIIV3, the latter was reported to increase 5-10 fold FVIII secretion (165, 189). In particular, gene therapy experiments using AAV carrying the FVIIIV3 resulted in a supraphysiologic FVIII expression up to 700% of normal in HA mice and reached 100% antigen levels in non-human primate models (165). In the future will be interesting for us to combine the pF8 ability to induce sustained FVIII expression with the use of codon optimized FVIII to improve gene therapy outcomes for hemophilia A treatment. Overall, this work provides new insight in extrahepatic sources of FVIII and in its transcriptional regulation. Other than to confirm FVIII production in endothelial cells and particularly in LSEC we demonstrated that also hematopoietic cells, mainly of myeloid origin, when transplanted and engraft express and secrete the protein as shown by phenotypic correction reached in HA mice. Moreover, pF8 study results well correlate with these findings showing its ability to drive transgene expression in LSEC, in spleen macrophages and generally in myeloid cells but less in lymphocytes by *in vivo* and *ex-vivo* LV delivery. Finally, by expressing FVIII under its promoter we were able to reach therapeutic FVIII levels in HA mice with limited immune response.

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